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MBS&amp;S 919 854-1401

NO. 3773 P. 2  
 Rec'd PCT/PTO 18 JUN 2001  
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FORM PTO-1350U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV. 6-95)		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		9250-37
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	U.S. APPLICATION NO. (If Exam. See 37 C.F.R. 1.6)
PCT/EP99/10179	December 16, 1999	PRIORITY DATE CLAIMED December 18, 1998
TITLE OF INVENTION		
HEPATITIS Y VIRUS		
APPLICANT(S) FOR DO/EO/US		
Sing Hiem Yap; Chris Julien Cornelius Verslype; Pieter Jacob Boender; Jan Albert Hellings		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))           <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))           <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>		
Items 11. to 16. below concern other document(s) or information included:		
<ol style="list-style-type: none"> <li>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.  <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input checked="" type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>16. <input type="checkbox"/> Other items or information: International Search Report; International Preliminary Examination Report; PCT Published Application WO 00/37650; PCT Chapter II Demand; PCT Request; Documentation supporting biological deposit with ECCC of deposit numbers 98121503, 98121504 and 98121505</li> </ol>		

Received from &lt;919 854 1401&gt; at 4/4/03 8:37:29 AM [Eastern Standard Time]

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MBS&amp;S 919 854-1401

NO. 3773 P. 3

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.50)		INTERNATIONAL APPLICATION NO. PCT/EP99/10179		ATTORNEY'S DOCKET NUMBER 9250-37	
17. <input type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00  International preliminary examination fee paid to USPTO (37 CFR 1.482). ..... \$670.00  No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$690.00  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO. .... \$970.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4). .... \$96.00 <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$840.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	14 -20 =	0	X \$18.00	\$	
Independent Claims	6 -3 =	3	X \$78.00	\$234.00	
Multiple dependent claim(s) (if applicable)			+ \$260.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$1,074.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
<b>SUBTOTAL =</b>				\$1,074.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$1,074.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
<b>TOTAL FEES ENCLOSED =</b>				\$1,074.00	
				Amount to be refunded	\$
				charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$1,074.00 to cover the above fees is enclosed.  b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 50-0220.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: "Express Mail" mailing label number EL733093137US Date of Deposit June 18, 2001  Myers Bigel Sibley & Sajovec Post Office Box 37428 Raleigh, North Carolina 27627					
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Signature: <i>Sorajini Biswas</i> Sorajini J. Biswas 39,071 REGISTRATION NUMBER					

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**09/868553**  
JC18 Rec'd PCT/PTO 1 8 JUN 2001

Attorney's Docket No. 9250-37

PATENT

**IN THE UNITED STATES DESIGNATED OFFICE (DO/US)**

In re: Application of Yap et al.  
Intl. Appln. No.: PCT/EP99/10179  
Intl. Filing Date: December 16, 1999  
For: HEPATITIS Y VIRUS

Date: June 18, 2001

DO/EO/US  
Commissioner for Patents  
Washington, DC 20231

**PRELIMINARY AMENDMENT**

Sir:

Prior to the examination of the above application, please amend the above-identified application as follows. Please enter the following amendments prior to the calculation of the filing fee.

**In the Specification:**

Please make the following amendments to the specification:

On page one of the application, after the Title of the Invention, please insert the following:

**Related Application Information**

This application claims priority under 35 U.S.C. § 371 from PCT Application No. PCT/EP99/10179 (published under PCT Article 21(2) in English), filed on December 16, 1999, which claims the benefit of EP Application No. 98204313.5, filed on December 18, 1998 and EP Application No. 99200167.7, filed on January 20, 1999, the disclosures of which are incorporated by reference herein in their entireties.

In re: Application of Yap et al.  
Intl. Appln. No.: PCT/EP99/10179  
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**In the Claims:**

Please delete Claims 3 and 9.

Please amend the claims to read as follows:

1. A method for detecting non-A-G hepatitis virus in a sample negative for Hepatitis C Virus (HCV), comprising contacting said sample with monoclonal antibody HCV.OT 1F and then detecting the presence or absence of immunoreactivity between the monoclonal antibody and the sample, wherein the presence of immunoreactivity indicates the presence of non-A\_G hepatitis virus.

2. An immortalized human hepatocyte cell line deposited at the European Collection of Cell Cultures (ECACC) under accession number 98121503.

4. An isolated Hepatitis y virus genome comprising a nucleotide sequence that hybridizes to a nucleic acid of the sequence of SEQ ID NO: 1 or to the complement thereof.

5. An isolated nucleic acid sequence comprising a nucleotide sequence that hybridizes to a nucleic acid having a sequence selected from the group consisting of SEQ ID NO: 1 or the complement thereof and the sequence of SEQ ID NO: 2 or the complement thereof.

6. An isolated polypeptide comprising an amino acid sequence or fragment thereof wherein said amino acid sequence is encoded by a nucleotide sequence that hybridizes to a nucleic acid having a sequence selected from the group consisting of SEQ ID NO: 1 or the complement thereof and the sequence of SEQ ID NO: 2 or the complement thereof.

7. An antibody reactive with a polypeptide according to claim 6 or a functional fragment thereof.

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8. A method for the detection of hepatitis Y virus in a sample comprising the steps of isolating nucleic acid from said sample, contacting the nucleic acid with a nucleotide sequence selected from the group consisting of SEQ ID NO: 1 or a fragment thereof, and SEQ ID NO:2 or a fragment thereof, and detecting the presence or absence of hybridization between the nucleic acid and the nucleotide sequence, wherein the presence of hybridization indicates the presence of hepatitis Y virus.

10. A method for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a subject suspected of being infected with Hepatitis Y virus, contacting the sample with a hepatitis Y virus according to claim 5 or a polypeptide according to claim 6, and then detecting the presence or absence of immunoreactivity between the sample and the virus or the polypeptide, wherein the presence of immunoreactivity indicates infection of the subject with hepatitis Y virus.

11. A method for diagnosing infection with hepatitis Y virus comprising the steps of contacting a sample from a subject suspected of being infected with Hepatitis Y virus, with an antibody according to claim 6 and then detecting the presence or absence of immunoreactivity between the monoclonal antibody and the sample, wherein the presence of immunoreactivity indicates the subject is infected with Hepatitis Y virus .

12. A method for growing Hepatitis Y virus (HYV) comprising infecting cells with HYV and propagating said cells in vitro, wherein the HYV comprises a nucleotide sequence that hybridizes with a nucleic acid selected from the group consisting of SEQ ID NO: 1 or the complement thereof, and SEQ ID NO:2 or the complement thereof .

13. A vaccine composition comprising a polypeptide according to claim 6 in a pharmaceutically acceptable excipient .

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14. A vaccine composition comprising a nucleic acid sequence according to claim 5 in a pharmaceutically acceptable excipient .

Please **add** the following claims:

15. A method of diagnosing non-A-G hepatitis, comprising infecting a cell of claim 2 with an inoculum from a subject suspected of having non-A-G hepatitis, contacting the cell with monoclonal antibody HCV.OT 1F, and then detecting the presence or absence of immunoreactivity between the monoclonal antibody and the cell, wherein the presence of immunoreactivity indicates the subject has non-A-G hepatitis.

16. The method according to claim 8, wherein the nucleotide sequence has the sequence of SEQ ID NO: 1 or a fragment thereof.

#### **REMARKS**

The pending claims have been amended above to better conform to United States practice. New claims 15 and 16 correspond respectively to the subject matter of Claim 3 and Claim 9, now deleted. The marked-up version of the changes to the specification and claims are attached hereto at pages 6-10, and are captioned "**MARKED UP COPY OF AMENDMENTS TO THE SPECIFICATION**" and "**MARKED UP COPY OF AMENDMENTS TO THE CLAIMS**," respectively.

It is submitted that this application is now in condition for substantive examination, which action is respectfully requested.

Respectfully submitted,



Sorajini J. Biswas  
Registration No. 39,111

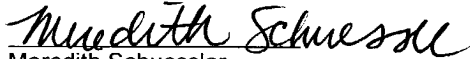
In re: Application of Yap et al.  
Intl. Appln. No.: PCT/EP99/10179  
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Meredith Schuessler  
Date: June 18, 2001

In re: Application of Yap et al.  
Intl. Appln. No.: PCT/EP99/10179  
Intl. Filing Date: December 16, 1999  
Page 6

**MARKED UP COPY OF AMENDMENTS TO THE SPECIFICATION**

On page one of the application, after the Title of the Invention, please insert the following:

**- - Related Application Information**

This application claims priority under 35 U.S.C. § 371 from PCT Application No. PCT/EP99/10179 (published under PCT Article 21(2) in English), filed on December 16, 1999, which claims the benefit of EP Application No. 98204313.5, filed on December 18, 1998 and EP Application No. 99200167.7, filed on January 20, 1999, the disclosures of which are incorporated by reference herein in their entireties.



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**MARKED UP COPY OF AMENDMENTS TO THE CLAIMS**

1 (Amended). A method for detecting non-A-G hepatitis virus in a sample negative for Hepatitis C Virus (HCV), [HCV wherein] comprising contacting said sample [is contacted with antibodies obtainable from a cell line deposited at the ECACC under accession number 9110711 or monoclonal antibodies having the same immunoreactivity] with monoclonal antibody HCV.OT 1F and then detecting the presence or absence of immunoreactivity between the monoclonal antibody and the sample, wherein the presence of immunoreactivity indicates [whereafter the presence of immune complexes formed is detected and from this] the presence of non-A\_G hepatitis virus [is determined].

2 (Amended). An [immortalised] immortalized human hepatocyte cell line deposited at the European Collection of Cell Cultures (ECACC) [ECACC] under accession number 98121503.

~~DELETED~~ [3. Use of the cell line according to claim 2 in the in vitro diagnosis of non-A-G hepatitis.]

4 (Amended). An isolated Hepatitis y virus genome comprising [characterised in that it comprises a genome comprising] a nucleotide sequence [hybridisable] that hybridizes to a nucleic acid of [to] the sequence of SEQ ID NO: 1 or [its] to the complement thereof.

5 (Amended). An isolated nucleic [Nucleic] acid sequence [derivable from the genome of Hepatitis y virus or from fragments of said genome or functional equivalents of said nucleic acid sequence, wherein Hepatitis Y virus is characterised in that it comprises a genome] comprising a nucleotide sequence that hybridizes to a nucleic acid having a sequence selected from the group consisting of [hybridisable to the sequence of] SEQ ID NO: 1 or [its] the complement thereof [or] and the sequence of SEQ ID NO: 2 or [its] the complement thereof.

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6 (Amended). **[A] An isolated** polypeptide comprising an amino acid sequence or fragment thereof wherein said amino acid sequence is encoded by a **[genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement,]** a nucleotide sequence **that hybridizes to a nucleic acid having a sequence selected from the group consisting of SEQ ID NO: 1 or the complement thereof and the sequence of SEQ ID NO: 2 or the complement thereof [or any functional equivalents of said polypeptide].**

7(Amended). **An antibody [Antibodies]** reactive with a polypeptide according to claim **[3] 6** or **a functional fragment [equivalents]** thereof.

8 (Amended). **A method [Method]** for the detection of hepatitis Y virus in a sample comprising the steps of isolating nucleic acid from said sample, **contacting the nucleic acid with a nucleotide sequence selected from the group consisting of SEQ ID NO: 1 or a fragment thereof, and SEQ ID NO:2 or a fragment thereof, and detecting the presence or absence of hybridization between the nucleic acid and the nucleotide sequence, wherein the presence of hybridization indicates the presence of hepatitis Y virus.**

**DELETED [9.** Method for the detection of hepatitis Y virus in a sample according to claim 5 characterised in that said nucleotide sequence is derived from the sequence in SEQ ID NO: 1.]

10 (Amended). **A method [Method]** for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a **[patient or animal] subject** suspected of being infected with Hepatitis Y virus, **contacting the sample with [providing]** a hepatitis Y virus according to **[claim 1] claim 5** or a polypeptide according to **[claim 3] claim 6, and then detecting the presence or absence of immunoreactivity between the sample and the virus or the polypeptide, wherein the presence of immunoreactivity indicates [and**

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**establishing immunoreactivity of said virus or polypeptide with antibodies in said sample] infection of the subject with hepatitis Y virus.**

11 (Amended). **A method [Method]** for diagnosing infection with hepatitis Y virus comprising the steps of **[providing] contacting** a sample from **[patient or animal] subject** suspected of being infected with Hepatitis Y virus, **[providing] with** an antibody according to **[claim 4] claim 6** and **then detecting the presence or absence of immunoreactivity between the monoclonal antibody and the sample, wherein the presence of immunoreactivity indicates the subject is infected with Hepatitis Y virus** [establishing whether immunoreactive components are present in said sample].

12 (Amended). **A method [Method]** for growing Hepatitis Y virus (**HYV**) comprising **[providing cells infected with HYV and] infecting cells with HYV and** propagating said cells in vitro, wherein **the** HYV [is characterised in that it **comprises a genome comprising] comprises** a nucleotide sequence **that hybridizes with a nucleic acid selected from the group consisting of SEQ ID NO: 1 or the complement thereof, and SEQ ID NO:2 or the complement thereof** [hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement].

13 (Amended). A vaccine composition comprising a polypeptide according to **[claim 3 in substantially isolated form mixed with] claim 6 in** a pharmaceutically acceptable excipient .

14(Amended). A vaccine composition comprising a nucleic acid sequence according to **[claim 2 in substantially isolated form mixed with] claim 5 in** a pharmaceutically acceptable excipient .

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**NEW CLAIMS:**

15. A method of diagnosing non-A-G hepatitis, comprising infecting a cell of claim 2 with an inoculum from a subject suspected of having non-A-G hepatitis, contacting the cell with monoclonal antibody HCV.OT 1F, and then detecting the presence or absence of immunoreactivity between the monoclonal antibody and the cell, wherein the presence of immunoreactivity indicates the subject has non-A-G hepatitis.

16. The method according to claim 8, wherein the nucleotide sequence has the sequence of SEQ ID NO: 1 or a fragment thereof.

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## HEPATITIS Y VIRUS

This invention is based on the discovery of a new form of human hepatitis, termed "Hepatitis Y". The invention relates to a viral agent associated with the disease, termed  
5 Hepatitis Y virus or HYV, and more in particular, relates to polynucleotide sequences derivable from this agent, polypeptides encoded by these polynucleotides, antibodies which specifically bind to these polypeptides and diagnostic methods and kits and vaccines that can be designed and made using these materials and the information that is embedded in these sequences. Moreover, the invention relates to an *in vitro* method for culturing HYV.

10 Hepatitis is one of the most important diseases transmitted from a donor to a recipient by transfusion of blood products, organ transplantation and hemodialysis; it also can be transmitted via ingestion of contaminated food and water, and by person-to-person contact. Viral hepatitis is known to be caused by a variety of viral agents with distinctive viral genes  
15 and modes of replication. Viral hepatitis is known to occur with differing degrees of severity of hepatic damage through different routes of transmission. In some cases, acute viral hepatitis can be clinically diagnosed by well-defined patient symptoms including jaundice, hepatic tenderness and an elevated level of liver transaminases such as aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH). In other cases, acute viral  
20 hepatitis may be clinically inapparent. The viral agents of hepatitis include hepatotropic viruses like Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), and systemic viruses like Hepatitis E virus (HEV), Epstein-Barr virus (EBV) and Cytomegalovirus (CMV). The latter class of viruses is not associated with chronic hepatitis

25 HAV infection occurs world-wide and is an important cause of acute viral hepatitis. HAV was discovered in 1973 (Feinstone et al. Science 1973;182:1026-8) and is a positive sense, single-stranded RNA virus classified within the genus hepatovirus of the picornaviridae family. With genomic RNA, HAV can function as messenger in directing the translation of  
30 proteins. A single large polypeptide is expressed from a large open-reading frame that extends through most of the genomic RNA. Translation takes place in a cap-independent fashion under control of an internal ribosome entry segment located within the 5' untranslated RNA. The polypeptide subsequently undergoes cleavage mediated by a viral protease, resulting in the

production of four capsid proteins and several non-structural proteins. HAV, which lacks a lipid envelope, is stable when it is secreted from infected liver cells into the bile, and it gains entry to the intestinal tract via this route. Thus, this virus typically spreads by a faecal-oral mode of transmission, and can cause extensive common-source outbreaks of disease. In countries where the disease is endemic, most of the population are infected during childhood. There are few or no symptoms, and the infected individuals acquire life-long immunity to the virus infection. The development of antibodies to HAV coincides with a marked diminution in quantity of viremia and faecal shedding of the virus (Lemon et al, J. Inf. Dis 1983;148:1033-9; Lemon et al. J. Inf. Dis. 1990;161: 7-13). Detection of this acute-phase antibody response is the mainstay of diagnosis. Antibodies to HAV of the IgM class are present in more than 99% of patients at the time of their initial presentation. The IgM antibody appears during acute illness or shortly after infection and persists for several months; its detection is a marker of a present or a recent infection. Thereafter, antibodies of the IgG class appear and remain life-long, providing immunity to re-infection (Dienstag and Isselbacher, In: Isselbacher KJ, Braunwald E, Wilson JD, eds. Harrison's Principles of Internal Medicine, 13th Ed. New York: McGraw-Hill, 1994, pp 1458-78; Ramos-Soriano and Schetwartz, Gastroenterol. Clin. North Am. 1994;23:753-67). Recombinant and inactivated HAV vaccines are now available and have been proven to be both safe and effective (Katkov and Dienstag, Gastroenterol. Clin. North Am 1995; 24:147-59.)

HBV infection is world-wide, one of the most frequent viral infections in humans. HBV is a partially double-stranded circular DNA virus that belongs to the family of hepadnaviruses (or hepatotropic DNA viruses). The discovery of "Australia antigen" by Blumberg in 1964 (Blumberg, Bull. NY Acad. Med. 1964; 40:377) has finally led to the finding of Dane particle as the causative agent of this type of hepatitis. The Dane particle is the complete HB virion composed of a partially double-stranded DNA genome including the nucleocapsid (hepatitis B core antigen - HBcAg), which is coated by the hepatitis B surface antigen (HBsAg) (Dienstag and Isselbacher, 1994; Ramos-Soriano and Schwartz, 1994). The first serological marker which appears after HBV infection is HBsAg. This viral antigen can be detected from 2 to 12 weeks after infection with HBV. The presence of HBsAg often antedates symptoms or abnormalities of liver biochemistry by 6-8 weeks. In acute resolving disease, it becomes undetectable 1 to 2 months following the onset of jaundice and rarely persists beyond 6

months. In chronic HBV infection, HBsAg remains detectable beyond 6 months. In acute resolving disease, after HBsAg disappears, antibodies to HBsAg (anti-HBs) become detectable in serum and remain life-long providing immunity to re-infection. The IgM antibody to HBcAg (anti-HBc IgM) appears usually 2 weeks after the detection of HBsAg, and it remains  
5 detectable for up to 6 months after onset of acute hepatitis. Before the disappearance of this antibody, another class of antibody to HBcAg (anti-HBcAg IgG) appears and remains life-long. The detection of the anti-HBc IgM is of assistance in diagnosing an acute or recent infection in patients with HBsAg concentrations that are below the sensitivity threshold of the diagnostic assay. Another important serological marker of HBV infection, the HBAg (hepatitis  
10 B antigen), appears concurrently with or shortly after the appearance of HBsAg in serum and is a marker of viral replication. In addition to the serological markers, serum HBV-DNA has been found to be the most sensitive marker of viral replication it can be detected very early during the course of infection, especially if it is determined by an amplification technique (Dienstag et al., 1994; Ramos-Soriano and Schwartz, 1994).

15

In the early 1970s, when specific diagnostic assays for detection of HAV and HBV infections became available, it was clear that some cases of enterally and of parenterally transmitted hepatitis were not caused by these agents or by any other known systemic viruses (CMV, EBV, HSV etc.). Such cases were classified as non-A, non-B viral hepatitis.

20

The aetiology of non-A, non-B viral hepatitis was an enigma until 1989-1990 when it became more transparent by the cloning of the HCV genome (Choo et al., Science 1989;244:359-62) and the isolation of a cDNA clone of the HEV (Reyes et al., Science 1990 247: 1335-9). HCV is identified as the major cause of parentally transmitted non-A, non-B hepatitis (Alter HJ et al., N. Engl. J. Med. 1989; 321:1494-1500; Weiner et al.; Lancet 1990;  
25 335: 1-3). At that time more than 50% of all cases of non-A, non-B chronic hepatitis was found to be related to HCV infection (Hammel et al., J. Hepatol.1994; 21: 618-23), while HEV was found as the main causative agent for enterally transmitted non-A, non-B viral hepatitis (Reyes et al., 1990; Bradley, Br. Med. Bull. 1990; 46:442-61; Velazquez, JAMA 1990; 263: 3281-5) especially in Asia and Africa

30

HCV, the major causative agent of post-transfusion non-A, non-B hepatitis, poses a serious world-wide health problem. HCV is a spherical enveloped virus of approximately 50

nm in diameter (Shimizu et al, 1996). The genome of HCV is a positive single-stranded RNA virus (Choo et al., 1989; Dienstag and Isselbacher, 1994; Houghton et al., 1991; Houghton, 1996) and classified as a member of the flaviviridae family. Its genome is unsegmented and is approximately 9.5 kb in size with a large open-reading frame encoding a polyprotein of 3011 amino acids long. The genomes of structural proteins are located at the 5' end and the non-structural genes are at the 3' end. The structural proteins consist of a capsid protein and two envelope proteins (E1 and E2). The non-structural proteins consist of at least six polypeptides, including protease, helicase, RNA polymerase enzymes and regulatory peptides. HCV is not a homogeneous virus population but it comprises a heterogeneous group of viruses (Dusheiko et al., 1996). Current patterns of hepatitis C virus classification are based on genetic relatedness. As with many RNA viruses. HCV is highly diverged - at least six major genotypes and multiple subtypes have been described (Ohno and Lau, 1996).

HCV infection can be determined by detection of antibodies targeted to HCV structural and non-structural proteins. Serological tests such as enzyme linked immunoassays (ELISA) are used primarily to screen blood donors and for diagnosis of HCV infection in symptomatic patients.

The anti-HCV antibodies, even those detected by third generation methods, are presumably not the neutralising antibodies as they can be detected in the serum of patients with ongoing HCV infection (Koziel, 1996). Detection of HCV-RNA necessitates amplification of the circulating viral RNA, e.g. by using reverse transcriptase polymerase chain reaction (RT-PCR). Methods of quantitating HCV genomes in the serum are also available.

Hepatitis C vaccine is not yet available. Sequence variation especially in the envelope region of the HCV genome, high rates of mutation, and an obscure humoral response are all challenges for the development of an effective HCV vaccine (Katkov et al. 1996).

Hepatitis D virus (HDV) is a transmissible agent that is defective and requires co-infection with HBV for viral replication. Molecular studies led to the identification of the genome of HDV. It has a small genome (1700 nucleotides), consisting of single-stranded RNA, and was circular with a high degree of base pairing. The genome of HDV is present in a HD antigen nucleocapsid that is coated with HBsAg. Co-infection with this virus can be demonstrated by the presence of IgM antibodies to the delta agent (anti-HDV) in addition to



the markers of HBV infection. Co-infection with HBV and HDV may cause fulminant hepatitis. Super-infection with HDV is marked by the presence of both IgM and IgG antibodies directed to HDV together with negative or low titres of IgM anti-HBc. Super-infection may cause a deterioration of a previous stable chronic HBV carrier state and may lead to a more rapid progression of liver disease (Ramos-Soriano and Schwartz, 1994). HDV-RNA can be detected in the serum by the principle of RT-PCR or by other amplification methods (Ramos-Soriano and Schwartz, 1994).

Hepatitis E is an enterally transmitted disease, similar to hepatitis A, that is spread primarily by faecal contaminated drinking water (Dienstag and Isselbacher, 1994; Ramos-Soriano and Schwartz, 1994; Prudy and Krawczynski, 1994). HEV is small, spherical, non-enveloped virus with morphologic and biophysical properties most similar to viruses found in the family of Caliciviridae. The genome of HEV is approximately 7.5 kb in length and consists of a positive-sense, single stranded RNA molecule that contains three distinct open reading frames (ORF1, ORF2, ORF3) that appear to encode for non-structural and structural proteins based on the presence of well-defined consensus motifs and genomic organisation similar to those of other Calici- or Calici-like viruses (Reyes et al., 1990).

The precise taxonomy of the virus has not yet been clarified (Koziel, 1996). A number of diagnostic tests for HEV infection have been developed over recent years. Immune electron microscopy using serum taken after acute HEV infection was used to detect aggregates of viral particles in stool samples (Bradley et al., 1987). In specimens obtained by liver biopsy, the fluorescent antibody-blocking assay detects HEV antigen present in hepatocytes (Krawczynski and Bradley, 1989). The cloning of HEV (Reyes et al., 1990) has led to the development of a number of ELISAs using recombinant HEV antigens (Dawson et al., 1992; Tsarev et al., 1993). The assay was based on the detection of antibodies to the ORF2 region, which contains sequences thought to code for virus capsid protein, and the ORF3 region, which overlaps the ORF1 and ORF2 region of viral RNA (Reyes et al., 1990). Anti-HEV of the IgM class appears first in the serum and thereafter anti-HEV IgG. HEV-RNA can be detected by RT-PCR in the stool of patients in the acute phase of hepatitis E (Koziel, 1996).

Although sensitive and specific tests for detection of the known hepatitis viruses are available, there still have been nearly 20% of patients with acute hepatitis of which the

etiology remains unexplained (Deka et al., 1994; Alter HJ and Bradley, 1995). Indeed, a number of clinical studies have suggested the existence of additional viral agents responsible for the occurrence of hepatitis. These include sporadic hepatitis (Marcellin et al., 1993); a substantial fraction of post-transfusion hepatitis (Alter HJ et al., 1989); community-acquired acute resolving and chronic hepatitis (Alter MJ et al., 1992; Buti et al., 1994); and fulminant hepatitis (Fagan et al., 1992; Kuwada et al., 1994). These non-A, non-B, non-C, non-E cases are further referred to as non-A-E hepatitis.

In 1994, Deka and colleagues claimed to have transmitted an enteric agent (designated as HFV) responsible for sporadic non-A-E hepatitis from human to rhesus monkeys using a patient's stool extract (Deka et al., 1994). Virus-like particles, 27-37 nm in diameter, were reported to be present in the infectious human stool extract as well as in the liver and stools of inoculated monkeys. The particles were shown to contain about 20 kilobases of double-stranded DNA. This finding has unfortunately not yet been confirmed by others until to date. More extensive studies will be required to confirm the existence of HFV.

In 1967, Deinhardt et al. Published serial transmission experiments in animal models with suspected infectious sera of patients with hepatitis of unknown etiology (Deinhardt et al., 1967). One of the infectious sera was obtained from a 34 year old surgeon with initials GB, collected during the third day of jaundice. He had no obvious exposure to serum hepatitis and had a history of moderate illness. Five serial marmoset-to-marmoset passages were carried out, and resulted in serum elevations of liver enzymes and histopathological changes in liver biopsies of almost all inoculated animals. The identification of the hepatitis A and B viruses in 1970's has excluded these two viral agents as the cause of hepatitis in this surgeon. Subsequent experiments have led to the further characterisation of the "hepatitis GB agent" (Almeida et al., 1976; Karayiannis et al., 1989).

Simons et al. have successfully cloned two flavivirus-like genomes from sera of tamarinds infected with the GB hepatitis agent (Simons et al., 1995a). Phylogenetic analysis showed that the sequences, which were designated as GB virus A and GB virus B, are neither  
30 genotypes of HCV nor genotypes of the same virus.

The genomes of GBV-A and GBV-B consist of 9493 nucleotides and 9143 nucleotides respectively (Muerhoff et al., 1995). Northern blot hybridisation showed that RNAs of GBV-B

but not of GBV-A could be detected in the liver of the inoculated tamarinds. GBV-B viremia caused a serum elevation of liver enzymes, whereas no enzyme elevation was observed in animals with only GBV-A viremia. Although nucleic acids of both viruses were present in plasma in acute phase, the exclusive evidence for the present of GBV-B in livers of infected animals suggests that GBV-B causes hepatitis in the inoculated tamarinds, while GBV-A seems only to be a transmissible virus in tamarinds without hepatitis inducing capacity by itself. However, further in vivo studies by Schlauder et al. (1995) demonstrated that the peaks of ALT-values of GBV-B infected animals were significantly lower than in GBV-A/GBV-B co-infected animals, suggesting that the severity of hepatitis in these animals is related to the presence of both viruses.

Efforts were subsequently made to detect antibodies in human sera by ELISA using recombinant proteins generated from GBV-A and GBV-B. Numerous immunoreactive sera were identified, but studies with RT-PCR using specific primers for these agents failed to confirm the presence of viral nucleic acids. The positive results of serological investigations might therefore have been due to cross-reactivities with proteins encoded by a related virus. Further investigations pointed out that GBV-A induces hepatitis in tamarinds but has probably no relevance in human (Schlauder et al., 1995).

Despite the above mentioned findings, further investigation of those immunoreactive sera with degenerate primers of the putative NS3 helicase region revealed a RT-PCR product with limited sequence identity to GBV-A, GBV-B and HCV. GENBANK analysis and alignment of the predicted translation product to other positive stranded RNA-viruses indicated that this sequence is derived from a novel member of the flaviviruses. This new virus was designated as GB virus C (GBV-C). The GBV-C viral genome consists of a positive sense, single-stranded RNA molecule of 9.2 kb. GBV-C encodes a putative single large polypeptide in which the structural proteins are positioned at the N-terminal end, and the non-structural proteins at the C-terminal end. Sequence analysis revealed that GBV-C was 59.0%, 47.9% and 53.7% identical at the nucleotide level, and 64.2%, 50.4% and 57.3% identical at amino acid level to GBV-A, GBV-B and HCV-1 sequences, respectively. These findings indicated that GBV-C is a member of the Flaviviridae family, more closely related to GBV-A than to GBV-B and clearly distinct from the representative member of the HCV family.

Parallel to the discovery of the hepatitis GB viruses, another flavivirus-like agent was identified. In January 1996, Linnen et al. reported the identification of an RNA virus that was designated as hepatitis G virus (Linnen et al., 1996). Further studies using an immunoreactive complementary DNA clone yielded the entire genome of 9392 nucleotides encoding a polyprotein of 2873 amino acids. The virus is closely related to GBV-C and distantly related to HCV, GBV-A, and GBV-B. This virus is a member of the flavivirus family and is associated with acute and chronic hepatitis in humans. This virus was detected in five of 38 patients (13%) with acute non-A-E hepatitis. None of the five patients developed chronic hepatitis but four remained HGV-RNA positive over a period of 2 to 9 years. In addition, HGV-RNA was detected in the serum of 18% of patients with acute hepatitis C, 1.7% of blood donors with normal serum alanine aminotransferase (ALT) levels, and 1.5% of blood donors with elevated ALT levels (>45 IU/L) (Linnen et al., 1996). The virus is found also to associate with both sporadic and post-transfusion hepatitis (Simons et al., 1995b; Linnen et al., 1996). HGV has also been detected in the serum of patients with non-A-E fulminant hepatitis and in patients with chronic non-A-E hepatitis (Yoshida et al., 1995; Dawson et al., 1996). It has a global distribution. Nevertheless, the role of this virus in the pathogenesis of liver injury is still not clear (Alter MJ et al., 1996).

From these findings, it is clear that HGV-RNA was not detected in many acute and chronic non-A, B, C cases even when using three different primer sets which are derived from the 5'-UTR, NS3 and NS5 regions of the HGV genome.. While the current assays may lack sensitivity, it is more likely that such cases are due to other hepatotropic agents. GBV-B may be responsible for some of the acute non-A-E hepatitis cases, but it seems that this viral agent is not associated with chronic hepatitis (Alter HJ et al., 1997; Alter MJ et al., 1997; Yashina et al., 1997).

Recently, the putative envelope protein (E2) of HGV has been cloned and expressed in Chinese hamster ovary cells (Tacke et al., 1997; Dille et al., 1997). This E2 viral protein is probably located on the surface of the virus, and has been assumed to be a target for human immune responses. An ELISA for detection of anti-HGV-E2 antibodies has been developed. Detection of antibodies to this protein is thought to be associated with a past HGV infection (Tacke et al., 1997; Dille et al. 1997).

In December 1997, a novel DNA virus was reported by Nishizawa et al. to be associated with elevated aminotransferase levels in the sera of 3 to 5 patients with biopsy-proven post-transfusion hepatitis of an at that time unknown aetiology. Association with elevated aminotransferase levels was reported by Nishizawa et al. This virus was designated TT-virus (TTV), after the initials of the first patient from whom the virus was isolated. TTV is an unenveloped single-stranded DNA virus for which a sequence of 3,739 bases was determined.

In subsequent analyses, evidence of potential hepatotropism of TTV was reported with TTV-DNA titers shown to be 10- to 100-fold greater in liver tissue than in serum. In addition, TTV-DNA titers correlated with aminotransferase levels in TTV-infected patients with non-A-G post-transfusion hepatitis. The relative hepatotropism and correlation of TTV titers with biochemical markers of hepatitis are in contrast to HGV, for which neither characteristic has been demonstrated.

In follow-up to the initial description of TTV (Okamoto et al., 1998 it was reported that 12% (34 of 290) of Japanese blood donors have detectable TTV DNA in their serum as measured by PCR. The prevalence of TTV in Japanese patients with chronic non-A-G hepatitis (and/or cirrhosis) was found to be 46% (41 of 90). Similar prevalences were reported for patients with risk factors for parenteral transmission of infectious agents, including intravenous drug users (40%), patients with haemophilia A (68%), and hemodialysis patients (46%). Clearly, with prevalences of this magnitude, TTV infection can produce liver disease in a minority of, if any, infected patients. However, it is possible that in a subset of TTV-infected patients, TTV may cause acute and/or chronic liver injury. This may be on the basis of host or viral characteristics, such as genotypic variations in TTV.

Two characteristics of post-transfusion TTV-infection make TTV a relatively attractive candidate virus as a potential cause of liver disease. The first of these is the observation that in TTV-infected patients with non-A-E post-transfusion hepatitis, the concentration of TTV is on average 10- to 100-fold higher in liver tissue than in serum. The second characteristic is that, in newly TTV-infected patients with non-A-E post-transfusion hepatitis, TTV titres rose and fell with aminotransferase levels, becoming undetectable in patients who normalised their aminotransferases. Neither of these characteristics, hepatotropism or correlation of viral titers with serum transaminases, were ever demonstrated for HGV. It should be kept in mind, however, that the higher prevalence of TTV infection in patients with cryptogenic cirrhosis

and idiopathic fulminant hepatic failure may simply reflect higher rates of parenteral and non-parenteral exposure to TTV than was experienced by the control groups.

As these recently described viruses at best seem to account for only a part of the non A-E hepatitis cases, it will be advantageous if means would be available to detect and characterise other non A-E hepatitis causing agents. This will allow for the development of specific diagnostic tests, anti-viral agents and preventive and therapeutic vaccines.

We undertook a prospective study in 165 patients who were admitted to the liver unit of the University Hospital in Leuven, Belgium for a liver biopsy because of acute or chronic liver disease. These patients underwent hepatological work-up including determination of serological markers of known hepatitis virus infection, autoantibodies for the diagnosis of autoimmune liver disease and tests for metabolic liver disease such as haemochromatosis and Wilson's disease. Data on anti-HCV and serum HCV-RNA were also collected from the past examinations and during follow-up in order to exclude intermittent viremia which may exist in seronegative chronic hepatitis C patients. In addition, a possible infection with HGV was also investigated by testing serum HGV-RNA using a PCR-assay. Liver biopsies were reviewed for histology and examined for granular immunoreactivity to the monoclonal antibody HCV.OT1F as described previously (Yap et al.,1994 , Example 1).

20

In summary, in this prospective study of 165 patients we have identified a large number of non-A-E, non-G hepatitis patients further referred to as non-A-G hepatitis patients (Example 1). Interestingly, liver biopsies from a large number of 121 patients from this study showed immunoreactivity with monoclonal antibody HCV-OT1F. When all HCV patients were excluded from this group, a number of 93 patients remained. This finding was unexpected since monoclonal antibody HCV-OT1F was raised against an amino acid sequence derived from HCV core antigen and previously thought to be rather specific for HCV (Yap et al.,1994). When this finding was further investigated it appeared that monoclonal antibody HCV-OT1F was capable of reacting with many more amino acid sequences than just the amino acid sequence of HCV against which it was raised (Example 3). Data obtained from a study based on a so-called replacement net of the HCV sequence against which the monoclonal antibody HCV-OT1F was raised, confirm this notion. Since the group

30

of non-A-G patients largely overlaps with the group of patients positive with monoclonal antibody HCV-OT1F but negative for any signs of HCV infection, it is concluded that immunological staining with monoclonal antibody HCV-OT1F might provide a useful tool in the diagnosis of a new form of non-A-G viral hepatitis.

5

The immunostaining method with monoclonal antibody HCV.OT1F also provides for a tool to detect transmission of such a new form of non-A-G viral hepatitis as it may occur in patients with a persisting hepatitis after orthotopic liver transplantation (OLT) who were liver HCV.OT1F immunoreactive, but remained serum HCV-RNA and anti-HCV negative during follow-up. A review of the viral and histological data of these patients before OLT, showed that HCV-OT1F liver immunoreactivity was found prior to OLT, despite anti-HCV and serum HCV-RNA were negative and remained negative after OLT. Finally, in one patient, HCV-OT1F immunoreactivity associated with chronic hepatitis was found after OLT while it was negative before OLT. This finding is strongly suggestive for a *de novo* infection, possibly by the route of blood transfusion. From these findings based on clinical studies of patients who underwent OLT, we can therefore conclude that anti-HCV negative, serum HCV RNA negative and liver HCV-OT1F immunoreactivity associated liver disease is caused by a transmissible agent (Example 2).

Disease could also be transmitted by inoculation of non-human primates like chimpanzee and rhesus monkey with serum, plasma, liver cells and/or PBMC obtained from a human patient or of other infected non-human primates (Example 4).

In the material obtained from the above non-A-G patients we discovered and characterised a new etiological hepatitis agent and termed it Hepatitis Y virus (HYV).

25

The invention described here provides nucleic acids and polypeptides derivable from the hepatitis Y etiological agent HYV. Moreover, the present invention also provides tools for diagnosing an infection with this virus in patients suspected of having a viral hepatitis as well as vaccines for the prevention of disease. The invention also provides a method for the in vitro culturing of HYV, as well as methods for determining the presence of the nucleic acid of this agent in materials like plasma, serum and liver tissue or isolated liver cells from HYV infected individuals or non-human primates like chimpanzee or rhesus monkey as well as from culture

30

supernatants or cell lysate of in vitro infected cell cultures. The invention also provides antigens derivable from the genome of hepatitis Y and antibodies reacting to these antigens.

The invention provides nucleic acids derivable from the genome of HYV. These sequences were obtained as described in Example 6. In brief: a cDNA difference analysis protocol, essentially as described by Hubank M. and Schatz, D. (NAR,22,5640-5648) is followed using nucleic acid from serum obtained from (a) non-A-G hepatitis patient(s) using human cot-1-DNA (Gibco), liver cDNA and human genomic DNA as the so-called driver in successive rounds of differential hybridisation (Example 6). Alternatively, plasma or liver from non-A-G infected patients may be used as a source of nucleic acid for this procedure. Enriched sequences were cloned into a vector and grown in an appropriate bacterial system to allow sequencing of the insert. Excluding known sequences, primers for PCR were designed for the sequences obtained. PCR applied to cDNA prepared from material including serum, plasma and liver cells from patients suspected to be infected with Hepatitis Y and controls allowed specific detection of hepatitis Y and isolation of further sequences specific for HYV. As an example of such sequences SEQ ID NO: 1 and SEQ ID NO: 2 are provided. (Example 6). Plasmids containing this sequence were deposited at the ECACC (accession numbers 98121504 and 98121505).

**SEQ ID NO: 1:**

	GATCACAAGC AACTGCCCGA CGAACGTACG CTGAGCGTAT TCGTCGACGA ACTGCACGCC	60
	CTCGACAAAC AGCGCCTGTC CGGCAAGCTG TCCGAGGAGT TCAACCGCGC CTATACCGGC	120
25	ATGTCCAGCG TGGCCAAAGC CACTGCCCGG CGCGTTGGCC GACTGGACGC CCAGGCGCTG	180
	CAAAGCCAAG GCGTGCAGAC GCTGCTCGAG GCCCACC GCA ACTGGAGCAA GCCCGAGCTG	240
30	TGGTACGCCA TCGAGCGCGC CGGCAAGGTT TACACCTACG ATTACTACCT GACCGCACTG	300
	GATC	304



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SEQ ID NO: 2

5 GATCGABGTG CAACACGCCC GCCTATHACG GCGCGTATTG CTTGTBGCAG CCTGAGTGCA 60  
 GCATTAGATT AGCCAATTAT CTGGGGCACC ATCATAAGCA GAAGGGATAA GCATGGCGCT 120  
 CACCGACCAA TCCACCCGCA CCGGTACCGG CGAAGAAGTC GACGCTGCCG TCATCGACGC 180  
 10 CTATCTCAAG GCCcATATTC CCGGCCTGAG TGGCGAGGCC GG 222

NOTE: in SEQ ID NO: 2, B = C, G or T; H = A, C or T; c = probably C

15 Cesium chloride equilibrium gradient centrifugation fractions were prepared from serum of a patient with Hepatitis Y or from high speed centrifugation-supernatant of liver homogenate from another patient with Hepatitis Y. Both were tested by nucleic acid amplification using nested primers according to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO:6 derived from SEQ ID NO: 1. Fractions with a density around 1.22 g/cm<sup>3</sup> were found to contain genomic sequences detectable by the method described in Example 7.  
 20 These fractions thus contain the purified virus particles. Accordingly, the invention provides a Hepatitis Y virus characterized in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

25 The invention thus provides a nucleic acid sequence derivable from the genome of Hepatitis Y virus or from fragments of said genome or functional equivalents of said nucleic acid sequence, wherein Hepatitis Y virus is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

30

Having available a probe for HYV as provided with SEQ ID NO: 1 or SEQ ID NO: 2, methods to characterize the HYV particle and its genomic nucleic acid are now further routine in the field and include sucrose gradient centrifugation to determine sedimentation coefficient, ultra filtration through controlled pore filters to determine particle size, disruption with  
 35 detergents and chaotropic agents to determine composition of the particle.

### The HYV Genome

Further polynucleotides from the genome of HYV may be obtained by routine methods,  
5 for example by producing a phage library from nucleic acid isolated from serum, plasma or  
liver from HYV positive humans or non-human primates or cell culture extracts and plate  
these phages to form plaques. The resulting phages can than be screened using SEQ ID NO: 1  
or its complement or the sequence of SEQ ID NO: 2 or its complement or fragments thereof as  
hybridization probes. Other hybridization probes or PCR primers can then easily be selected.

10 Hybridization probes and primers for nucleic acid amplification methods are also part of  
the invention. The use of these for the further characterization of the genome of Hepatitis Y  
etiological agent is described in Example 9. For this purpose a total nucleic acid preparation  
created by hexamer primed first- and second strand cDNA synthesis on serum nucleic acids,  
15 tissue culture extracts or purified viral material using standard procedures is used as input  
material.

In a first round this preparation is subjected to a-specific amplification by anchor primer  
addition through blunt-end ligation in which the anchor primer has a specific restriction site  
and a subsequent PCR reaction with the anchor primer. After restriction digestion of the PCR  
20 products, these products are cloned in a phage vector and plaques created with the  
recombinant phages are screened by hybridization with a HYV specific probe.

Alternatively, anchor linkers, consisting of a long and a short arm that are both  
unphosphorylated, can be added to the ends of the cDNA products after restriction digestion  
with a restriction enzyme compatible with the overhang of these primers. After non-specific  
25 amplification with anchor-complementary primers specific amplification can be performed by  
ligating these PCR products in a cloning vector (by specific restriction or through T/A  
cloning) and performing a PCR reaction on this ligation reaction using a combination of  
vectorprimers situated on both sides of the insert and one of two primers situated on the  
complementary strand of a double stranded HYV specific sequence. The resulting PCR  
30 products can be enriched further for HYV specific sequences by ligating these PCR products  
in a vector and performing a PCR on this ligation reaction using a combination of vector  
primers situated on both sides of the insert and one of two primers situated on the

complementary strand of a double stranded HYV specific sequence but downstream of the primers in the preceding PCR reaction. These PCR products can be cloned, screened with HYV specific primers situated within the region bounded by the restriction site used and the HYV specific primer used in the final amplification step.

5 Another approach is by making use of the Marathon cDNA procedure (Clontech). In this procedure double stranded cDNA is ligated with a specially designed adaptor sequence of which the shorter sequence is blocked. This sequence will therefore not be elongated and is displaced by the product made by elongating the HYV specific primer. The resulting PCR products can be screened further by one of the procedures above or a combination of these.

10

Segmentation of the genome can be detected by Northern blotting. If the viral genome is unsegmented, the complete genome can be isolated applying one or more of the procedures described above.

15 The extreme 5'- and 3'-ends of the genome are isolated by ligating the genomes head to tail together and isolating the junction fragments by PCR using as primers the sequences situated on the ends of the established sequence.

20 If the viral genome is segmented, and on the assumption that different segments have sequence motif in common, cDNA synthesis and PCR amplification as described above is followed by cloning in a vector and hybridization screening with the complete HYV specific sequence.

Otherwise, if the segments do not have motifs in common, a new RDA experiment can be performed using the known HYV sequence as complementary driver sequence.

25 **HYV encoded Peptides**

The invention also provides polypeptides encoded by the HYV genome. From the sequences of SEQ ID NO: 1 the potential amino acid sequence can be read in six frames as provided in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16

30

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SEQ ID NO 11:

Ser Gln Ala Thr Ala Arg Arg Thr Tyr Ala Glu Arg Ile Arg Arg Arg  
1 5 10 15  
5 Thr Ala Arg Pro Arg Gln Thr Ala Pro Val Arg Gln Ala Val Arg Gly  
20 25 30  
Val Gln Pro Arg Leu Tyr Arg His Val Gln Arg Gly Gln Ser His Cys  
10 35 40 45  
Pro Ala Arg Trp Pro Thr Gly Arg Pro Gly Ala Ala Lys Pro Arg Arg  
50 55 60  
15 Ala Asp Ala Ala Arg Gly Pro Pro Gln Leu Glu Gln Ala Arg Ala Val  
65 70 75 80  
Val Arg His Arg Ala Arg Arg Gln Gly Leu His Leu Arg Leu Leu Pro  
85 90 95  
20 Asp Arg Thr Gly  
100

SEQ ID NO: 12:

Ile Thr Ser Asn Cys Pro Thr Asn Val Arg Xaa Ala Tyr Ser Ser Thr  
1 5 10 15  
25 Asn Cys Thr Pro Ser Thr Asn Ser Ala Cys Pro Ala Ser Cys Pro Arg  
30 20 25 30  
Ser Ser Thr Ala Pro Ile Pro Ala Cys Pro Ala Trp Pro Lys Pro Leu  
35 35 40 45  
Pro Gly Ala Leu Ala Asp Trp Thr Pro Arg Arg Cys Lys Ala Lys Ala  
50 55 60  
Cys Arg Arg Cys Ser Arg Pro Thr Ala Thr Gly Ala Ser Pro Ser Cys  
40 65 70 75 80  
Gly Thr Pro Ser Ser Ala Pro Ala Arg Phe Thr Pro Thr Ile Thr Thr  
85 90 95  
45 Xaa Pro His Trp Ile  
100



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SEQ ID NO: 15:

Ile Gln Cys Gly Gln Val Val Ile Val Gly Val Asn Leu Ala Gly Ala  
1 5 10 15  
5  
Leu Asp Gly Val Pro Gln Leu Gly Leu Ala Pro Val Ala Val Gly Leu  
20 25 30  
10  
Glu Gln Arg Leu His Ala Leu Ala Leu Gln Arg Leu Gly Val Gln Ser  
35 40 45  
Ala Asn Ala Pro Gly Ser Gly Phe Gly His Ala Gly His Ala Gly Ile  
50 55 60  
15  
Gly Ala Val Glu Leu Leu Gly Gln Leu Ala Gly Gln Ala Leu Phe Val  
65 70 75 80  
Glu Gly Val Gln Phe Val Asp Glu Tyr Ala Gln Arg Thr Phe Val Gly  
85 90 95  
20  
Gln Leu Leu Val Ile  
100

SEQ ID NO: 16:

25  
Ser Ser Ala Val Arg Xaa Xaa Ser Xaa Val Xaa Thr Leu Pro Ala Arg  
1 5 10 15  
30  
Ser Met Ala Tyr His Ser Ser Gly Leu Leu Gln Leu Arg Trp Ala Ser  
20 25 30  
Ser Ser Val Cys Thr Pro Trp Leu Cys Ser Ala Trp Ala Ser Ser Arg  
35 40 45  
35  
Pro Thr Arg Arg Ala Val Ala Leu Ala Thr Leu Asp Met Pro Val Xaa  
50 55 60  
Ala Arg Leu Asn Ser Ser Asp Ser Leu Pro Asp Arg Arg Cys Leu Ser  
65 70 75 80  
40  
Arg Ala Cys Ser Ser Ser Thr Asn Thr Leu Ser Val Arg Ser Ser Gly  
85 90 95  
45  
Ser Cys Leu Xaa  
100

NOTE: in SEQ ID NO 11 -SEQ ID NO:16 Xaa denotes a termination codon

Other peptides may easily be obtained by expression cloning. In this method, phages are prepared from a library as described above using the methods of Huynh et al. (In: D. Glover (ed.), DNA Cloning: A Practical Approach, IRL Press Oxford, 49-78, 1985). The phages are plated to form plaques and the plaques are screened for production of poly-peptides immunoreactive with HYV serum. The serum used for screening the plaques can be from any HYV source including human, chimpanzee or rhesus monkey serum. Alternatively, immunogenic oligopeptides may be deduced from the nucleic acid sequences obtained as described above by methods widely known in the field. Once an amino acid sequence is known, immunogenic and antigenic fragments thereof may be obtained by methods described below. From the nucleic acid sequences SEQ ID NO:1 or the sequence of SEQ ID NO: 2 or other HYV sequences, one or more reading frames can be deduced. Overlapping oligopeptides can be synthesized and screened for immunoreactivity with HYV sera.

Thus, the invention provides a polypeptide comprising an amino acid sequence or fragment thereof wherein said amino acid sequence is encoded by a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement, or any functional equivalents of said polypeptide.

Also included within the scope of the present invention are nucleic acid sequences derivable from functional variants of (fragments of) the HYV genome as well as functional variants of the encoded amino acid sequences as for instance are shown in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16.

It will be understood that for a particular polypeptide derived from HYV, natural variations can exist between individual viruses or strains. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions from which can be expected that they do not essentially alter biological and immunological activities, have been described. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and

structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous polypeptides. Nucleic acid sequences encoding such homologous functional variants are included within the scope of this invention. Moreover, the potential exists to use recombinant DNA technology for the preparation of nucleic acid sequences encoding these various functional variants.

The information provided in SEQ ID NO: 1 or SEQ ID NO:2 allows a person skilled in the art to isolate and identify the nucleic acid sequences encoding the various functional variant polypeptides mentioned-above having corresponding immunological characteristics with the protein specifically disclosed herein. The generally applied Southern blotting technique or colony hybridization can be used for that purpose (Experiments in Molecular Biology, ed. R.J. Slater, Clifton, U.S.A., 1986; Singer-Sam, J. et al., Proc. Natl. Acad. Sci. 80, 802-806, 1983; Maniatis T. et al., Molecular Cloning, A laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, USA, 1989). For example, restriction enzyme digested DNA fragments derived from a specific HYV strain is electrophoresed and transferred, or "blotted" thereafter onto a piece of nitrocellulose filter. It is now possible to identify the nucleic sequences encoding the functional variant polypeptides on the filter by hybridization to a defined labelled DNA or "probe" back translated from the amino acid sequences shown in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16, under specific conditions of salt concentration and temperature that allow hybridization of the probe to any homologous DNA sequences present on the filter. After washing the filter, hybridized material may be detected by autoradiography. Once having identified the relevant sequence, DNA fragments that encode a functionally variant polypeptide to the polypeptide disclosed in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16 can now be recovered after agarose gel electrophoresis.

A nucleic acid sequence according to the invention may be isolated from a particular HYV strain and multiplied by molecular biology methods including recombinant DNA techniques and polymerase chain reaction (PCR) technology or may be chemically synthesized in vitro by techniques known in the art.



A preferred nucleic acid sequence according to the invention is characterized in that said sequence contains at least part of the DNA sequence disclosed in SEQ ID NO:1 or its complement.

5 As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in an other codon but still coding for the same amino acid, e.g. both GAT and GAA are codons for the amino acid glutamic acid. Consequently, it is clear that for the expression of a polypeptide with the amino acid sequence shown in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16 use can be  
10 made of a variant nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in SEQ ID NO:1.

Furthermore, also fragments of the nucleic acid sequences encoding the specifically disclosed protein or functional variants thereof as mentioned above are included in the present invention. All modifications resulting in the above-mentioned functional variants of the  
15 specifically exemplified polypeptide are included within the scope of the present invention.

The term "fragment" as used herein means a DNA or amino acid sequence comprising a subsequence of the nucleic acid sequence or polypeptide of the invention. Said fragment is or encodes a polypeptide having one or more immunogenic determinants of a particular HYV  
20 protein, i.e. has one or more epitopes of the HYV protein reactive with a given antibody or capable of eliciting an immune response in a suitable host. Fragments can inter alia be produced by enzymatic cleavage of precursor molecules, using restriction endonucleases for the DNA and proteases for the polypeptides. Other methods include chemical synthesis of the fragments or the expression of polypeptide fragments by DNA fragments.

25

An "immunogenic fragment" is understood to be a fragment of the full-length protein that has retained its capability to induce an immune response in the host. An "immunoreactive fragment" is understood to be a fragment of the full length protein that has retained its capability to react with an antibody. At this moment, a variety of techniques is available to  
30 easily identify DNA fragments encoding immunogenic or immunoreactive fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent Application WO 86/06487, US Patent nr. 4,833,092, Proc. Natl Acad. Sci. 81: 3998-

4002 (1984), J. Imm. Meth. 102, 259-274 (1987), the so-called PEPSCAN method is an easy to perform, quick and well-established method for the detection of epitopes; the immunologically important regions of the protein, used world-wide and as such well-known to man skilled in the art. This (empirical) method is especially suitable for the detection of B-cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific polypeptide fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. 78: 38248-3828 (1981)), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47: 45-148 (1987) and US Patent 4,554,101). T-cell epitopes can likewise be predicted from the sequence by computer with the aid of Berzofsky's amphiphilicity criterion (Science 235, 1059-1062 (1987) and US Patent application NTIS US 07/005,885). A condensed overview is found in: Shan Lu on common principles: Tibtech 9: 238-242 (1991), Good et al on Malaria epitopes; Science 235: 1059-1062 (1987), Lu for a review; Vaccine 10: 3-7 (1992), Berzowsky for HIV-epitopes; The FASEB Journal 5:2412-2418 (1991)

Two nucleic acid fragments are considered to have hybridisable sequences if they are capable to hybridising to one another under typical hybridisation and wash conditions, as described, for example in Maniatis, et al., pages 320-328, and 382-389, or using reduced stringency wash conditions that allow at most about 25-30% basepair mismatches, for example: 2x SSC, 0.1% SDS, room temperature twice, 30 minutes each, then 2x SSC, 0.1% SDS 37 °C once, 30 minutes; then 2X SSC, room temperature twice ten minutes each. Preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches. These degrees of homology can be selected by using wash conditions of appropriate stringency for identification of clones from gene libraries or other sources of genetic material, as is well known in the art.

Two amino acid sequences or two nucleic acid sequences are considered homologous if they have an alignment score of more than 5 (in standard deviation units) using the program ALIGN with the mutation gap matrix and a gap penalty of 6 or greater (Dayhoff). The two sequences (or parts thereof, preferably at least 35 amino acids in length) are homologous if

their amino acid alignments are greater than or equal to 40% preferably 60% and more preferably 80% using the ALIGN program mentioned above.

A DNA or cDNA fragment is derivable from the Hepatitis Y genome if it has the same or substantially the same basepair sequence as a cloned region of a Hepatitis Y genome

### **Antibodies against HYV**

Polyclonal and monoclonal antibodies, either purified from a natural host including human, chimpanzee or rhesus monkey infected by HYV or raised in experimental animals against HYV polypeptides or other structural elements are also part of the invention.

Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogeneous binding characteristics for the relevant antigen. Homogeneous binding as used herein refers to the ability of the antibody species to bind to ligand binding domain of the invention.

The antibody is preferably a monoclonal antibody, more preferably a humanised monoclonal antibody.

Monoclonal antibodies can be prepared by immunizing inbred mice, preferably Balb/c with the appropriate protein by techniques known in the art (Kohler and Milstein, Nature 256; 495-497, 1975). Hybridoma cells are subsequently selected by growth in hypoxanthine, thymidine and aminopterin in an appropriate cell culture medium such as Dulbecco's modified Eagle's medium (DMEM). Antibody producing hybridomas are cloned, preferably using the soft agar technique of MacPherson, (Soft Agar Techniques, Tissue Culture Methods and Applications, Kruse and Paterson, eds., Academic Press, 276, 1973). Discrete colonies are transferred into individual wells of culture plates for cultivation in an appropriate culture medium. Antibody producing cells are identified by screening with the appropriate immunogen. Immunogen positive hybridoma cells are maintained by techniques known in the art. Specific monoclonal antibodies are produced by cultivating the hybridomas in vitro or preparing ascites fluid in mice following hybridoma injection by procedures known in the art.

It is preferred to use humanized antibodies. Methods for humanizing antibodies, such as CDR-grafting, are known (Jones et al., Nature 321, 522-525, 1986). Another possibility to

avoid antigenic response to antibodies reactive with polypeptides according to the invention is the use of human antibodies or fragments or derivatives thereof.

Human antibodies can be produced by in vitro stimulation of isolated B-lymphocytes, or they can be isolated from (immortalized) B-lymphocytes which have been harvested from a human being infected with HYV or immunized with at least one polypeptide according to the invention.

It is also preferred to use single chain antibodies. Methods for the production of such antibodies are known in the art.

The invention thus provides antibodies reactive with a polypeptide encoded by the genome of hepatitis Y virus or functional equivalents thereof

#### **Diagnostic assays for HYV**

Diagnostic assays based on the use of nucleic acid amplification and/or nucleic acid hybridization are also part of the invention. The primers and probes can be designed using methods known in the art, for instance based on SEQ ID NO: 1, SEQ ID NO: 2 or other sequences derived from DNA sources as described in Example 7 and 9. An example of a set of primers based on SEQ ID NO:1 is given in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO:6, examples of primers based on SEQ ID NO: 2 are provided in SEQ ID NO: 7, SEQ ID NO: 8 ,SEQ ID NO:9. and SEQ ID NO: 10,

SEQ ID NO: 3           CGTACGCTGAGCGTA

SEQ ID NO: 4           GGCGTACCACAGCTC

SEQ ID NO: 5           CACGCCCTCGACAAACAGCG

SEQ ID NO: 6           TCTGCACGCCTTGTTTGCA

SEQ ID NO: 7           GTGCAACACGCCCCGC

SEQ ID NO: 8           CCGGCCTCGCCACTC

SEQ ID NO: 9           ACGGCGCGTATTGCTTGT

SEQ ID NO: 10          GCCGGGAATATGGGCCTT

The method described in Example 7 typically allows detection of the HYV genome in clinical samples like serum or plasma. The results show that 7 out of 10 non-A-G patients were positively identified as carrying the HYV genome in their serum or liver. Zero out of 6 control subjects were positive. More details are given in Example 7.

5

Standard methods of epitope prediction can be used to select the most likely immunoreactive regions as described herein. Synthetic peptides representing immunogenic, immunoreactive or functional domains of the HYV related polypeptides can be prepared by standard methods known in the art. Such oligopeptides as well as polypeptides produced by expression of recombinant DNA in prokaryotic or eukaryotic systems are also part of the invention

10

Another preferred embodiment of the present invention is directed to a method for the detection of antibodies to HYV, the method comprising contacting the sample suspected to contain antibodies to hepatitis Y virus particles or antigens with at least one peptide according to the present invention.

15

Yet another preferred embodiment of the present invention is directed to the detection of nucleic acid from HYV or related to HYV. This method may comprise contacting the sample with a suitable nucleic acid probe or probes and detecting binding of the probe or probes. Alternatively, the nucleic acid from the sample may first be amplified in a specific or non-specific way. Many suitable methods for these purposes are known in the art.

20

In *in vitro* assays, some form of supports is often used to immobilise the binding molecules or peptides according to the present invention. Supports which can be used are, for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an ceramic particle (such as a ceramic magnetizable particle with active aldehyde surface groups), an erythrocyte, a dye sol, a metal sol or metal compound as sol particle, a carrier protein such as BSA or KLH.

25

30

Also some form of labeling is often used to detect the antigen-antibody or hybridisation interaction. Labeling substances may be radioactive or non-radioactive such as a radioactive isotope, a fluorescent compound, a chemiluminescent compound, an enzyme, a dye sol, metal sol or metal compound as sol particle. Depending upon the format of the assay, either the specific binding molecules within the scope of the invention can be labeled, or other specific binding molecules, which bind to them are labeled. Immunoassays (including radioimmunoassays) and immunometric assays (including immunometric radioassays and enzyme-linked immunosorbent assays) can be used, as can immunoblotting techniques.

*In vitro* assays may take many formats. Some depend upon the use of labeled specific binding molecules such as antibodies (whose use is included within the scope of the invention), whereas some detect the interaction of antibody (or other specific binding molecule) and antigen by observing the resulting precipitation. These are well known in the art.

*In vitro* assays will often be conducted using kits. According to yet another aspect of the present invention, there is provided an assay kit for the detection of hepatitis Y viral particle or antigen, the kit comprising a specific binding molecule as described above and means for detecting whether the specific binding molecule is bound to a hepatitis Y particle, antigen or nucleic acid.

The assay methodology may for example be any of the assays referred to above. Competitive and, especially, sandwich immunoassay kits are preferred. The specific binding molecule and the detection means may be provided in separate compartments of the kit. The specific binding molecule may be provided bound to a solid support. The detection means may comprise a detectable labeled second specific binding molecule (which itself may be an antibody (monoclonal but preferably polyclonal), which bind to the bound hepatitis Y particle or antigen.

Also preferred are kits for the detection of nucleic acid from or associated with HYV. Such kits may comprise nucleic acid primers for the amplification of HYV sequences such as the primers in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO:6 and optionally a suitable probe to detect the amplified product.

Another preferred embodiment of the present invention to an assay kit for the detection of antibodies to hepatitis Y virus, the kit comprising at least one peptide according to the present invention and means for detecting whether the peptide is bound to antibodies to hepatitis Y virus.

5 A test kit for carrying out a sandwich reaction for the detection of antibodies to hepatitis Y virus may comprise, for example, a peptide according to the invention coated to a solid support, for example the inner wall of a microtest well, and either a labeled peptide according to the invention or a labeled anti-antibody.

For carrying out a competition reaction, the test kit may comprise a peptide according to  
10 the invention coated to a solid support, and a labeled specific binding molecule according to the invention.

The invention thus provides for a method for the detection of hepatitis Y virus in a sample comprising the steps of isolating nucleic acid from said sample and hybridising said  
15 nucleic acid with a nucleotide sequence derived from the genome of hepatitis Y virus.

The invention also provides for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a patient or animal suspected of being infected with Hepatitis Y virus, providing a hepatitis Y virus or a polypeptide derived from hepatitis Y virus  
20 or functional equivalent thereof and establishing immunoreactivity of said virus or polypeptide with antibodies in said sample.

The invention also provides a method for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a patient or animal suspected of being  
25 infected with Hepatitis Y virus, providing an antibody against hepatitis Y virus derivable polypeptides and establishing whether immunoreactive components are present in said sample.

#### **Vaccines against HYV**

30 Immunogenic preparations according to the invention can also be used to produce vaccines against infection with HYV and the resulting disease. Antibodies reactive with these

immunogenic preparations can be selected for their neutralising capacity in the infectivity assay using the in vitro culture of the "C-cell line" (ECACC deposit number 98121503)

These antibodies can be used as passive prophylaxis or therapeutic preparation aiding in removing HYV from the infected host. The peptides according to the present invention can also be used in a vaccine for the treatment of HYV infection.

In addition to an immunogenically effective amount of the active peptide the vaccine may contain a pharmaceutically acceptable excipient.

The immunogenicity of the peptides of the invention, especially the oligopeptides, can be enhanced by cross-linking or by coupling to an immunogenic carrier molecule (i.e. a macromolecule having the property of independently eliciting an immunological response in a patient, to which the peptides of the invention can be covalently linked) or if part of a protein.

Covalent coupling to the carrier molecule can be carried out using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the peptides of the invention can be coupled, e.g. using water soluble carbodiimides such as dicyclohexylcarbodiimide, or glutaraldehyde.

Coupling agents such as these can also be used to cross-link the peptides to themselves without the use of a separate carrier molecule. Such cross-linking into polypeptides or peptide aggregates can also increase immunogenicity.

Examples of pharmaceutically acceptable excipients useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. mannose, sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F® or Marcol 52®), saponins or vitamin-E solubilisate.

The vaccine according to the present invention can be given inter alia intravenously, intraperitoneally, intranasally, intradermally, subcutaneously or intramuscularly.



The useful effective amount to be administered will vary depending on the age and weight of the patient and mode of administration of the vaccine.

The vaccine can be employed to specifically obtain a T cell response, but it is also possible that a B cell response is elicited after vaccination. If so, the B cell response leads to the formation of antibodies against the peptide of the vaccine, which antibodies will be directed to the source of the antigen production, i.e. the viral particle or cells expressing viral antigens on their surface. This is an advantageous feature, because in this way the cells are combatted by responses of both immunological systems.

Both immunological systems will even be more effectively triggered when the vaccine comprises the peptides as presented in an MHC molecule by an antigen presenting cell (APC). Antigen presentation can be achieved by using monocytes, macrophages, interdigitating cells, Langerhans cells and especially dendritic cells, loaded with one of the peptides of the invention or loading with protein including peptide or manosylated protein. Loading of the APC's can be accomplished by bringing the peptides of the invention into or in the neighbourhood of the APC, but it is more preferable to let the APC process the complete antigen. In this way a presentation is achieved which mimicks the *in vivo* situation the most realistic. Furthermore the MHC used by the cell is of the type which is suited to present the epitope.

An overall advantage of using APC's for the presentation of the epitopes is the choice of APC cell that is used in this respect. It is known from different types of APC's that there are stimulating APC's and inhibiting APC's.

Preferred are the listed cell types, which are so-called 'professional' antigen presenting cells, characterized in that they have co-stimulating molecules, which have an important function in the process of antigen presentation. Such co-stimulating molecules are, for example, B7, CD25, CD40, CD70, CTLA-4 or heat stable antigen (Schwartz, 1992, *Cell* 71, 1065-1068).

Fibroblasts, which have also been shown to be able to act as an antigen presenting cell, lack these co-stimulating molecules.

Instead of a vaccine with cells, which next to the desired expression products, also harbour many elements which are also expressed and which can negatively affect the desired immunogenic reaction of the cell, it is also possible that a vaccine is composed with liposomes which expose MHC molecules loaded with peptides, and which, for instance, are filled with lymphokines. Such liposomes will trigger a immunologic T cell reaction.

By presenting the peptide in the same way as it is also presented in vivo an enhanced T cell response will be evoked. Furthermore, by the natural adjuvant working of the, relatively large, antigen presenting cells also a B cell response is triggered. This B cell response will a.o. lead to the formation of antibodies directed to the peptide-MHC complex. This complex is especially found in tumor cells, where it has been shown that in the patient epitopes are presented naturally, which are thus able to elicit a T cell response. It is this naturally occurring phenomenon which is enlarged by the vaccination of APC's already presenting the peptides of the invention. By enlarging not only an enlarged T cell response will be evoked, but also a B cell response which leads to antibodies directed to the MHC-peptide complex will be initiated.

The vaccines according to the invention can be enriched by numerous compounds which have an enhancing effect on the initiation and the maintenance of both the T cell and the B cell response after vaccination.

In this way addition of cytokines to the vaccine will enhance the T cell response. Suitable cytokines are for instance interleukins, such as IL-2, IL-4, IL-7, or IL-12, GM-CSF, RANTES, MIP- $\alpha$ , tumor necrosis factor and interferons, such as IFN- or the chemokines.

In a similar way antibodies against T cell surface antigens, such as CD2, CD3, CD27 and CD28 will enhance the immunogenic reaction.

Also the addition of helper epitopes to stimulate CD4<sup>+</sup> helper cells or CD8<sup>+</sup> killer cells augments the immunogenic reaction. Alternatively also helper epitopes from other antigens can be used, for instance from heat shock derived proteins or cholera toxin.

Another type of vaccination having a similar effect is the vaccination with pure DNA, for instance the DNA of a vector or a vector virus having the DNA sequence encoding the peptides according the present invention (both homologues and heterologues (chimeric

protein) or repetitive). Once injected the virus will infect or the DNA will be transformed to cells which express the antigen or the peptide(s).

5 The invention thus provides a vaccine composition comprising a polypeptide derivable from hepatitis Y virus in substantially isolated form mixed with a pharmaceutically acceptable excipient

10 The invention also provides for a vaccine composition comprising a nucleic acid sequence derivable from hepatitis Y virus in substantially isolated form mixed with a pharmaceutically acceptable excipient

#### **In Vitro culturing of HYV**

15 The infectivity assay described in Example 5 can also be used to test antiviral substances such as nucleoside-analogues, enzyme inhibitors, agents inhibiting attachment and endocytosis of HYV to and into the host cell. The use of the assay for this purpose is also part of the invention (see Example 5).

20 The in vitro infection system in the "C-cell line" can be infected by HYV as shown in the examples and clones can be selected in which the HYV infectious particle is constitutively produced without causing disruption of the cell culture. The infection can be followed by several methods described herein, including performing nested RT-PCR with primers derived from SEQ ID-1 or other sequences produced according to the invention and also including immunostaining with monoclonal antibody HCV-OT1F.

25 HYV infectious particles and polypeptides and polynucleotides produced in this way are also part of the invention.

30 The invention thus provides for a method for growing Hepatitis Y virus (HYV) comprising providing cells infected with HYV and propagating said cells in vitro, wherein HYV is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

Analysis of the polypeptide and polynucleotide structure of HYV allows the  
5 identification of structural and functional targets for antiviral strategies, including nucleoside  
analogues, enzyme inhibitors, structural mimicry molecules and other natural or non-natural  
substances that can prevent or cure infection with HYV or the resulting disease. The in vitro  
infectivity assay can be used to monitor the effectiveness of such substances

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**FIGURES**

Figure 1. Recurrence of anti-HCV negative, serum HCV-RNA negative and liver HCV.OT 1F immunoreactive chronic aggressive hepatitis in a patient who underwent OLT for anti-HCV negative, serum HCV-RNA negative and HCV.OT 1F immunoreactive associated terminal liver cirrhosis.

Figure 2 : Serum aminotransferases (AST and ALT) and liver HCV.OT 1F immunoreactivity before and after inoculation (time 0) of chimpanzee Sylvia.

Figure 3 : Serum aminotransferases (AST and ALT) and liver HCV.OT 1F immunoreactivity before and after inoculation (time 0) of rhesus monkey I FU.

Figure 4 : Aminotransferase (ALT) values before and after inoculation of rhesus monkey 8925.

Figure 5 Cesium chloride gradient centrifugation of plasma of patient OHN

Figure 6 Cesium chloride gradient centrifugation of supernatant fraction S3

**EXAMPLES****EXAMPLE 1****CLINICAL CHARACTERISTICS OF PATIENTS WITH HEPATITIS Y**

5 Anti-HCV negative, HCV-RNA negative chronic non B-hepatitis associated with immuno-histochemical reactivity for a monoclonal antibody HCV.OT 1F

Previously we have reported the successful detection of hepatitis C virus core antigen by  
10 a monoclonal antibody HCV.OT 1F (, developed against a peptide with the sequence RTQQRKTKRSTNRRR (according to EP 0537856) which has 8 of 15 aminoacids in common with the sequence of the HCV core conserved region (according to EP 0388232) ) in liver biopsy specimens of patients with chronic hepatitis C, who were positive for anti-HCV and serum HCV-RNA (Yap SH et al., 1994. J Hepatol: 20 ; 275-81). The cytoplasmic granular  
15 immunoreactivity was absent in the cytoplasm of hepatocytes of patients suffering from various liver diseases, who were not suspected for infection with non-A, non-B agents. However, the typical immunoreactivity was also detected in the liver cells of a limited number of patients with chronic hepatitis B, suspected for infection with non-A, non-B agents, despite there was a lack of anti-HCV or serum HCV-RNA.

20 This striking finding can be considered as false positivity but it can also indicate that the HCV.OT 1F-staining is more sensitive for detection of HCV-infection than the classical assays. In addition, the finding may also be the result of HCV.OT 1F cross-reactivity to a unique agent associated with chronic non C-hepatitis. In an attempt to identify additional cases with chronic hepatitis and lacking of anti-HCV and serum HCV-RNA, but HCV.OT 1F  
25 immunostaining reactive in liverbiopsies, we initiated a prospective study in patients who were admitted to our liver unit for a liver biopsy because of acute or chronic liver disease. These patients underwent hepatological work-up including determination of serological markers of known hepatitis virus infection, autoantibodies for the diagnosis of autoimmune liver disease and tests for metabolic liver disease, such as haemochromatosis and Wilson's  
30 disease. Data on anti-HCV and serum HCV-RNA were also collected from the past examinations and during follow-up in order to exclude intermittent viremia which may exist in seronegative chronic hepatitis C patients. In addition, a possible infection with HGV was also

investigated by detection of serum HGV-RNA using PCR-assay. Liver biopsies were reviewed for histology and examined for granular immunoreactivity to the monoclonal antibody HCV.OT 1F.

From the 165 consecutive patients investigated so far, forty-two patients (25.5 %) were negative for anti-HCV, serum HCV-RNA and for liver HCV.OT 1F immunoreactivity (group A). Twenty-four patients (14.5 %) were positive for anti-HCV, serum HCV-RNA and for HCV.OT 1F immuno-staining (group B). Ninety-three patients (56.3 %) were liver HCV.OT 1F immunoreactive but lacking of anti-HCV and serum HCV-RNA (group C). In addition, a limited number of patients had a inconsistent pattern of anti-HCV, serum HCV-RNA and HCV.OT 1F immunoreactivity in the liver biopsies (table 1).

Table 1. The results of a prospective study for liver HCV.OT 1F immunoreactivity in 165 consecutive patients who underwent a liver biopsy because of acute or chronic liver disease and the relation with HCV serological markers.

	Number of patients	Anti-HCV	Serum HCV-RNA	Liver HCV.OT 1F immunoreactivity
<b>Group A</b>	42	negative	negative	negative
<b>Group B</b>	24	positive	positive	positive
<b>Group C</b>	93	negative	negative	positive
	2	negative	positive	positive
	2	positive	negative	positive
	1	positive	negative	negative
	1	positive	positive	negative

Most of these patients (> 95 %) underwent a liver biopsy because of elevated serum liver enzymes. Some patients had a biopsy for diagnosis of haemochromatosis or for the existence of a malignant tumor. In addition, a limited number of liver specimens were recruited for investigation from post-mortem donor livers, which were for some reasons not suitable for transplantation.

The elevated serum liver enzymes in patients from group A could be attributed to various liver disorders, as shown in table 2. The prevalence of hepatitis B infection in this group of patients was 12 %, and an association with suspected excessive alcohol intake was found in 12 % of the cases.

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**Table 2. Histological findings of liver biopsies of patients from group A (anti-HCV negative, serum HCV-RNA-negative and HCV.OT 1F immuno-non-reactive patients; total number : 42).**

10

	Drug-induced hepatitis (5)
	Donorliver, no signs of hepatitis (8)
	Cholangiocarcinoma (1)
15	Hepatitis B virus infection (1 acute, 2 chronic aggressive hepatitis, 2 active cirrhosis)
	Ito-cell hyperplasia (vit. A related) (1)
	Steatosis (2)
	Cryptogenic cirrhosis (1)
	Necrotizing hepatitis (4)
20	Autoimmune chronic hepatitis (1)
	Adenocarcinoma (1)
	Vanishing bile duct disease (1)
	PBC (2)
	Primary haemochromatosis (2)
25	Chronic active hepatitis (1)
	Minimal hepatitis (2)
	Liver metastasis (1)
	Granulomatous hepatitis (1)
	Caroli disease (1)
30	Nodular regenerative hyperplasia(1)
	Normal liver parenchyma next to presumed focal lesion (1)

All patients from group B with diagnosis of chronic hepatitis C had elevated serum liver enzymes. More than 90 % of the patients from group C had also abnormal serum liver enzymes at least two weeks before the liver biopsy was taken. The histological findings of the liver biopsies of patients from group C are shown in table 3. More than 75 % of the patients had a variable degree of portal and/or periportal inflammation. The association with hepatitis B virus infection was 11,8 %, which was comparable with the findings in group A. Thirty-one

35

patients were tested for the presence of serum HGV-RNA and only one was found to be positive. There was a suspicion of excessive alcohol intake in 27 patients (28 % in group C, as compared to the 12 % in group A. However, the degree of portal and periportal inflammation present in these liver biopsies could not be attributed solely to alcohol induced liver injury. It was suspected that additional viral infection might play a role.

**Table 3. Histological findings of patients from group C (anti-HCV negative, serum HCV RNA negative and liver 1F2-immunoreactive patients; total number : 93).**

10	Portal and/or periportal inflammatory infiltrates (29)
	Intralobular inflammatory infiltrates (6)
	Steato-hepatitis (15)
	Steatosis (4)
	Cirrhosis, hepatocellular carcinoma and portal inflammatory infiltrates (2)
15	Active cirrhosis and hepatitis B virus infection (3)
	Chronic hepatitis B virus infection (7)
	Chronic hepatitis B virus infection and steatohepatitis (1)
	Inactive cirrhosis (4)
	Primary biliary cirrhosis (1)
20	Ductopenia (1)
	Cirrhosis and steatohepatitis (9)
	Metastasis of a neuroendocrine tumor (1)
	Primary hemochromatosis and portal inflammatory infiltrates (3)
	Cirrhosis and hepatitis G virus infection (1)
25	No obvious abnormalities (6)

It is also interesting to note that 5 patients from group C had extrahepatic pathology : polymyositis (in one patient), periarteritis nodosa (1), mixed connective tissue disease (1), extrinsic allergic alveolitis (1) and one patient with an acute episode of fever, arthralgias, urticaria and eosinophilia.

Thirteen patients in group C had a follow-up of more than 3 years. Seven showed a persistent rise in serum liver enzymes, while 6 had a transient pattern. Seven out of ten patients with a follow-up of 1 to 3 years had persistent elevated serum liver enzymes and 3 had a transient rise. Normalisation of serum ALT was noted in one patient, who remained liver HCV.OT 1F immuno-reactive when he underwent a new liver biopsy because of the development of hepatocellular carcinoma.



From this prospective study of 165 patients who were admitted to the liver unit for liver biopsy because of acute or chronic liver disease we have identified a group of 93 patients with elevated serum liver enzymes and associated with histological findings of portal and/or periportal inflammation and a granular cytoplasmatic staining with monoclonal antibody HCV.OT 1F. These patients however, repeatedly showed the absence of anti-HCV and serum HCV-RNA.

Previously, we have described a similar finding but in a limited number of patients in a previous report (Yap SH et al., 1994 see above). All patients had concomittant hepatitis B virus infection, but were also suspected for a coinfection with non A, non B agents. In the present study of 93 patients the prevalence of HBV infection was 12 % which was comparable to the figure found in group A patients who were negative for anti-HCV and serum HCV-RNA and liver HCV.OT 1F immuno-non-reactive. Nevertheless, there is an association with a history of excessive alcohol intake in group C patients as compared to the group A. The liver histology of these patients showed however, pronounced portal and/or periportal inflammation which can not be attributed to alcohol intake. The liver histology is in fact compatible with the findings in chronic hepatitis C patients. Another particular feature of these patients is the frequent finding (29 patients) of steatosis or steatohepatitis.

From this study, we may therefore conclude that apart from HCV infection, monoclonal antibody HCV.OT 1F has identified a unique form of hepatitis non -A to G, which may be caused by a transmissible agent.

### Laboratory procedures

#### *Assay for liver enzymes*

Serum aminotransferases (sALT and sAST), alkaline phosphatases and gamma-glutamyl transferases were assessed using routine laboratory procedures.

#### *Assay for anti-HCV antibodies*

HCV antibodies were detected by a second generation anti-HCV enzyme-linked immunosorbent assay from Abbott (MEIA, AxSYM) and Sanofi (Monolisa). In some patients the Chiron RIBA HCV 3.0 SIA was used as a confirmatory test.

*RNA extraction.* RNA was extracted from 200 µl serum or EDTA treated plasma, in a single step acid guanidinium thiocyanate-phenol-chloroform extraction procedure, as described by Chomczynski & Sacchi (1987). After isopropanol precipitation, the RNA was dissolved in 8 µl DEPC-treated water.

5

HGV- and HCV-RNA detection by RT-PCR assay. For reverse transcription, the RNA was denatured by incubation for 10 min at 60°C. To the RNA fraction, 12 µl reverse-transcription mix was added, containing 200 units Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco BRL, Bethesda, MD), 30 units placental RNase inhibitor (Pharmacia, Uppsala, Sweden), 80 pmol random primers (Boehringer Mannheim, Germany), 0.5 mM of each deoxynucleotide triphosphate (dNTP; Pharmacia), 10 mM dithiotreitol, an appropriate volume of 5 x reverse transcriptase buffer, and DEPC treated water. The cDNA synthesis was carried out at 37°C for 1 hour and stopped by heating in boiling water for 1 minute. Subsequently, single round, double round nested or semi-nested PCR was performed.

10

Briefly, the first PCR mixture contained 5 µl cDNA solution, 20 pmol (HGV) or 50 pmol (HCV) of each primer, 0.2 mM of each dNTP and 1 unit Primezyme DNA polymerase (Biometra, Göttingen, Germany) in a total volume of 50 µl PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.01% gelatin). Samples were overlaid with mineral oil and subjected to thermal cycling. When nested or semi-nested PCR was performed, 3 µl of first round PCR product was added to a new PCR mixture containing the appropriate nested or semi-nested PCR primers for a second amplification. Amplified DNA was visualized by agarose-gel electrophoresis (2%) and ethidium bromide staining.

20

HGV-RNA was detected by single round PCR with primers specific to the non-structural (NS) 3 region (HGV-NS3.S : 5'GGTGAGATTCCCTTCTATGGGCATGG; HGV-NS3.A : 5'CCTCAGCAGTAGTGGAACAGGATTCGG) or by semi-nested PCR with primers for the 5' non-coding region (NCR) (HGV-NCR.S : 5'CGGCCAAAAGGTGGTGGATG; HGV-NCR.A1: 5'GGTTTAACGACGAGCCTGACG; HGV-NCR.A2: 5'CGGTAGG-GCCAACACCTGTGG where A1 is the anti-sense primer for the first round and A2 for the second round amplification) or the NS5 region (HGV-NS5.S: 5'CTCTTTGTGGTAGTAGCCGAGAGAT; HGV-NS5.A1: 5'TGAGTCAGAGGACGGGGTATCC; HGV-NS5. A2: 5'ATCTGAGCTGCTCTCGGTAACCG where A1 is the anti-sense primer for the first round and A2 for the second round amplification). Cycling conditions were 1 min 95°C, 1 min 55°C, 1 min 72°C. For single round PCR 45 cycles were

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performed, for semi-nested PCR, 45 and 30 cycles were performed for the first and second round amplification respectively.

HCV-RNA was detected by nested PCR with primers for the 5'NCR (first round: HCV-NCR.S1 : 5'CCACCATAGATCTCTCCCCTGT; HCV-NCR.A1 : 5'ATACTCGA  
5 GGTGCACGGTCTACGAGACCT; second round : HCV-NCR.S2 : 5'AGATCTTCA-CGCAGAAAGCGT; HCV-NCR.A2: 5'CACTCTCGAGCACCTATCA GGCAGT). First round cycling conditions were 96°C for 30 seconds, 48°C for 45 seconds, 72°C for 1 minute, 35 cycles. Second round cycling conditions were 96°C for 30 seconds, 42°C for 45 seconds, 72°C for 1 minute, 30 cycles.

10

#### Immuno-histochemistry

Immuno-histochemistry was performed as described previously (Yap SH et al., 1994, see above) on 4-µm-thick cryostat sections of fresh frozen materials using a three-step indirect immuno-peroxidase procedure. Sections were incubated overnight at 4°C with the monoclonal  
15 antibody HCV.OT 1F (purified IgG1 : 20 ng/µl). The secondary and tertiary antibodies consisted of peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit IgG, respectively (both obtained from Dakopats a/s Copenhagen, Denmark; working dilution 1/50 and 1/100, respectively). Each incubation was performed for 30 minutes at room temperature and followed by a wash in three changes of phosphate buffered saline, pH 7.2.  
20 The reaction product was developed by incubation for 15 min in 100 mM acetate buffer (pH 5.2), containing 0.05 % 3-amino-9-ethyl-carbazole and 0.01 % H<sub>2</sub>O<sub>2</sub>, resulting in bright red staining of immuno-reactive sites.

In order to abolish a weak "non-specific" background reactivity, the monoclonal antibody was pre-incubated overnight with the homogenate of normal human liver tissue  
25 before use.

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## EXAMPLE 2

### **Clinical evidence for the existence of a transmissible agent causing non-A-G hepatitis associated with HCV.OT 1F-liver immunoreactivity**

In order to demonstrate that non-B, non-C chronic hepatitis associated with HCV.OT 1F liver immunoreactivity is caused by a transmissible agent, we investigated patients who underwent an orthotopic liver transplantation. The setting of an orthotopic liver transplantation

(OLT) may represent an excellent in vivo model for demonstration of the transmissibility of a putative infectious agent causing chronic hepatitis.

For this study six patients (1-3, 6-8) who were HCV.OT 1F liver immunoreactive and underwent OLT were included. One patient was negative for HCV.OT1F immunostaining and in 2 patients the immunostaining was not performed before OLT. .

The serological markers of HCV infection and HCV.OT 1F liver immunoreactivity of these patients are shown in table 4.

**Table 4. Serological markers of HCV-infection and liver HCV.OT 1F immunoreactivity in patients who underwent an orthotopic livertransplantation.**

Patient	Indication for OLT	Markers for HCV infection			Liver histological findings after OLT
		<i>Before OLT</i>	<i>Liver donor</i>	<i>After OLT</i>	
1	Cirrhosis Terminal liver disease	anti-HCV + HCV-RNA + HCV.OT 1F +	anti-HCV - HCV-RNA - HCV.OT 1F -	anti-HCV + HCV-RNA + HCV.OT 1F +	Persisting hepatitis at 2 months
2	Cirrhosis + hepatocellular carcinoma	anti-HCV + HCV-RNA + HCV.OT 1F +	anti-HCV -	anti-HCV + HCV-RNA + HCV.OT 1F +	Persisting hepatitis at 6 and 9 months
3	Cirrhosis Terminal liver disease	anti-HCV + HCV-RNA	anti-HCV -	anti-HCV + HCV-RNA + HCV.OT	Cellular rejection at 1 week

		<b>+</b> <b>HCV.OT</b> <b>1F +</b>		<b>1F +</b>	
<b>4</b>	<b>Cirrhosis</b> <b>Terminal liver</b> <b>disease</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b>	<b>not known</b>	<b>anti-HCV +</b> <b>HCV-RNA</b> <b>+ HCV.OT</b> <b>1F +</b>	<b>Persisting hepatitis</b> <b>for 7 years</b>
<b>5</b>	<b>Cirrhosis</b> <b>Terminal liver</b> <b>disease</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b>	<b>not known</b>	<b>anti-HCV +</b> <b>HCV-RNA</b> <b>+ HCV.OT</b> <b>1F +</b>	<b>Persisting hepatitis</b> <b>for 7 years</b>
<b>6</b>	<b>Cirrhosis</b> <b>Terminal liver</b> <b>disease</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b> <b>HCV.OT</b> <b>1F +</b>	<b>anti-HCV -</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>- HCV.OT</b> <b>1F +</b>	<b>Persisting hepatitis</b> <b>for 3 years</b>
<b>7</b>	<b>Primary</b> <b>Biliary</b> <b>Cirrhosis</b> <b>Terminal liver</b> <b>disease</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b> <b>HCV.OT</b> <b>1F +</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b> <b>HCV.OT</b> <b>1F -</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>- HCV.OT</b> <b>1F +</b>	<b>Persisting hepatitis</b> <b>at 3 and 12 months</b>
<b>8</b>	<b>Fulminant</b> <b>liver failure</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b> <b>HCV.OT</b> <b>1F +</b>	<b>anti-HCV -</b>	<b>anti-HCV -</b> <b>HCV-RNA-</b> <b>HCV.OT</b> <b>1F +</b>	<b>Transient hepatitis</b> <b>at 2 months; normal</b> <b>biopsy at 1 year (and</b> <b>1 F-2 negative)</b>
<b>9</b>	<b>Primary</b> <b>Biliary</b> <b>Cirrhosis</b> <b>Terminal liver</b> <b>disease</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b> <b>HCV.OT</b> <b>1F -</b>	<b>anti-HCV -</b> <b>HCV-RNA</b> <b>-</b> <b>HCV.OT</b> <b>1F -</b>	<b>anti-HCV -</b> <b>HCV-RNA-</b> <b>HCV.OT</b> <b>1F +</b>	<b>Persisting hepatitis</b> <b>at 1 year</b>

Patients 1-5 were anti-HCV and serum HCV-RNA positive after OLT. All the patients in this group developed biochemical and histological changes of chronic hepatitis after OLT. Patient 1-3 had a HCV reinfection. These patients were positive for anti-HCV, serum HCV-RNA and HCV.OT 1F immunoreactivity before OLT. The liver donors of these patients were anti-HCV negative. In addition, the donor liver of the first patient before OLT was shown to be HCV-RNA negative and HCV.OT 1F immuno-non-reactive.

Case 4 and 5 were anti-HCV and serum HCV-RNA negative before OLT. These patients were infected by HCV during or some time after OLT.

Patient 6-8 were negative for anti-HCV and for serum HCV-RNA but HCV.OT 1F liver immunoreactive before OLT. Despite the anti-HCV and serum HCV-RNA remained negative, the liver grafts became HCV.OT 1F immunoreactive at some time after OLT and remained immunoreactive during follow-up. Patient 6 developed a chronic aggressive hepatitis (figure 1). In patient 7 only a mild persisting hepatitis was noted. Patient 8 was transplanted for fulminant liver failure associated with HCV.OT 1F liver immunoreactivity and the transplanted liver became HCV.OT 1F positive 2 months after OLT. One year after OLT the liver enzymes of this patient were normalized, and no HCV.OT 1F immunoreactivity could be observed in the liver biopsy specimen.

Patient 9 was negative for anti-HCV, serum HCV RNA and liver HCV.OT 1F immuno-non-reactive before OLT and developed a HCV.OT 1F positive chronic persisting hepatitis, with fluctuating liver enzymes after OLT. She remained anti-HCV and HCV-RNA negative for 1 year after OLT.

All liver donors of the patients 6-9 were anti-HCV negative. In addition, the liver biopsy specimens of patient 7 and 9 two hours after reperfusion of the new grafts were HCV-RNA negative, and HCV.OT 1F immuno-non-reactive. No specimens of donor livers were available for investigation from patient 6 and 8.

For patients who underwent an OLT as shown in this study, HCV.OT 1F immunostaining in liver biopsies could be a sensitive marker for reinfection (patient 1-3) or *de novo* infection (patient 4-5) of hepatitis C virus. These findings are in good agreement with the

previously reported good sensitivity and specificity of HCV.OT 1F immunostaining in patients with HCV infection (Yap S.H. et al., 1994. J Hepatol; 20 :275-81).

However, in this study we identified 3 patients (patient 6-8) with a persisting hepatitis after OLT who were liver HCV.OT 1F immunoreactive, but remained serum HCV-RNA and anti-HCV negative during follow-up. A review of the viral and histological data of these patients before OLT, showed that HCV.OT 1F liver immunoreactivity was found prior to OLT, despite anti-HCV and serum HCV-RNA were negative and remained negative after OLT. Finally, in one patient (patient 9), HCV.OT 1F immunoreactivity associated with chronic hepatitis was found after OLT while it was negative before OLT. This finding is strongly suggestive for a *de novo* infection.

From these findings based on clinical studies of patients who underwent OLT, we can therefore conclude that anti-HCV negative, serum HCV RNA negative and liver HCV.OT 1F immunoreactivity associated liver disease is caused by a transmissible agent.

### EXAMPLE 3

#### REACTIVITY PATTERN OF MONOCLONAL ANTIBODY HCV.OT 1F

A total of 4550 random linear dodecapeptides (12-mers), synthesized in credit-card format minipepscan cards, were screened with mAb HCV.OT 1F (1/100) as described previously (Slootstra et al., 1995, Molecular Diversity 1, 87-96)

##### 1. Summary of Results

The results are given in Table 1 below. The results are ranked for Optical Density (OD) values and the actual OD value found is followed by the sequences of the peptide in the respective test well.

##### *Alignment top 25 peptides*

The consensus tripeptide CXR, with CHR and CNR in particular, is a dominant sequence motif in the top 25 peptides

##### *Analysis of single amino acids*



In the top 200 peptides the frequency of the amino acids C and R is significantly higher compared to that of the other 18 amino acids

#### *Analysis of dipeptide motifs*

5       The highest scoring dipeptide motifs contain the amino acids C and/or R. Flanking high scoring amino acids include A, F, K, M and Q.

#### *Analysis of tripeptide motifs*

10       The occurrence of all possible 8000 tripeptides (000) as well as all possible spaced tripeptides (OXXXXOXO, OXOXO etc. etc.) in the top 200 peptides was determined. Most of the top tripeptides contain the sequence motif CXR. Flanking high scoring amino acids include A, F and M ).

#### *Positional analysis of CXR motif*

15       The sequence motif CXR has a preferred location in the second half of the peptide sequence.

### 2. Conclusions

20       The various analyses revealed that CHR or CNR plus the flanking residues A, F, K, M and Q are involved in antibody binding. We propose the following short sequences as candidate energetic cores of a mimotope: ACHR, ACNR, FCHR, FCNR, CHRF, CNRF, CHRXA, CNRXA, MXCHR, MXCNR, FXCHR, FXCNR (X, various residues). We expect that these motifs form a firm basis for the development of useful mimotopes.

25

Another important conclusion is that none of the most reactive peptides has a significant homology to the peptide the monoclonal antibody HCV.OT 1 F was raised against ( RTQQRKTKRSTNRRR), except for the relatively high frequency of occurrence of the aminoacid Arginine ( R ).

TABLE 5

TOP 100 REACTIVE 12-MER PEPTIDES FROM A 4550 RANDOM PEPTIDE  
LIBRARY, TESTED WITH MONOCLONAL ANTIBODY HCV.OT 1F

5 Peptides are ranked based on Optical density. The actual OD value is given followed by the sequence of the peptide in the respective test well.

	2919	TINTETCARAAV
	2676	MILRACHRQLCV
10	2574	RNMSKTSASAVE
	2557	EEKKQCNFTKLD
	2461	DMCKTIWACRRI
	2431	RHYVLCNRGTA
	2410	CYCWRCARVMFN
15	2313	QIMAEPRQTCNY
	1989	SEWAFCMRFIHQ
	1677	YRVQHCRRQRDT
	1652	HTSFSCMRDVFM
	1502	CNRPMYFKIPYI
20	1450	TERSFWMACHR
	1435	QCHRVAYIGGCN
	1420	IVYLDICKRRQ
	1381	RSRADQSSQKAC
	1377	ASILCLRISHQT
25	1377	KVLIVSCNRHKT
	1335	WMVACHLRLMSD
	1246	FPCLMLICKRFF
	1225	QSQVHMIEKNRD
	1206	AGASDKRLCNKI
30	1151	GTNHRNCWRLDQ
	1143	IFVQVQFACNIN
	1077	VEKTAFNCNVM

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55

	1073	RYQWNLPCNIMR
	1062	NPICQRFCAWK
	1049	SNDGCQFDILPK
	1038	TWGPSNQKTSKL
5	1018	MKMIWRDVQCNK
	956	ALELYAMRQCFC
	944	YCCRLAHQNCKF
	937	LMMHIVLWLCNK
	922	LQCFICRQSPAN
10	919	TMFCLRRSCTRS
	886	HDEACCRFFTHE
	876	TGQNLRHCRAMC
	870	AGYACKFSREGE
	868	IRCDIIYCWRQC
15	842	GDDWSHGCLRVF
	823	MEQAVQIHCFRQ
	819	GDEWTQPRFCQR
	791	EPEKNIERCAIA
	791	AAMTFYCHRTAF
20	789	DNLLWEVCDRYM
	785	KWNGYHEMPCNQ
	759	DYYTMNCERKEN
	758	RGLRWIFRRCFR
	756	TEWVCFRRWPTA
25	739	QELHEPIKCIRL
	738	CSDVVETRCQID
	738	QQYSCSCNTQLD
	722	GVFGICHFEKMA
	721	KRMCKNMQECGK
30	716	SFMCRWDISDRQ
	712	QQRWCLRTVMEY
	696	CFCAMVIRSKKA

5	691	VWALRCSRIRDH
	691	ACKFCQNGQSQM
	687	GMELNWDHHKQR
	681	ENFFVVGGETVAC
	681	QTMGGPSCQGIM
10	676	KGAMMRVRWTAC
	676	TRHGRHVPTRRC
	674	WDFWMQKEKGKI
	671	RCMYDVAYVTCT
	671	LMGNKMRCRMDR
15	665	WKTAYMPPNRLD
	662	WHETCMYQVFNQ
	661	SQRMWKENVMAT
	651	CCRDRIKVEDYA
	651	CACTIHWCFCCR
20	648	HPQGATNKMEGF
	645	CGLSNCDQTCHR
	645	IHKMTFGQMCLN
	636	EQPWKNQDFKLS
	634	WEAVVGFCARKI
25	628	ITWQWKNRAVFS
	628	LRVCHAPVSEMC
	627	PWPFCHIHAEFA
	627	FKCNMMEQNHVI
	625	YDWWQHNLTSDV
30	625	QHTKASWLQRTC
	622	MKVRCVDWRVVA
	620	HQFIARWQVWER
	620	MKGMYTDDAILE
	620	YMLKQKCSEQIC
	619	YFCADYIMQNSA
	614	CDYWGVFWKYNI

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614 YCNTLPDDFITK  
614 DIRQNWMTWCSR  
610 CPQVTESMHQLD  
610 HEQRRCKIVHKI  
5 610 PYGNRVSNLTSL  
608 KVWGIPFWVNAM  
603 QKCAYGFLILTP  
603 NCTEGHAVILMQ  
602 HFQGHQGVNWNV  
10 602 PRHKDVECMGQ  
602 QCAKYADLCYLT

#### EXAMPLE 4

15

Transmission studies in primates

##### 1. Introduction

20

From previous clinical studies of patients who underwent orthotopic liver transplantation, we demonstrated that anti-HCV negative, serum HCV RNA negative and liver HCV.OT 1F immunoreactivity associated liver disease is most likely caused by a transmissible agent. In order to determine whether this type of liver disease can also be transmitted from human to animals, we initiated transmission studies in primates. Additional  
25 objectives of the current project were (1) To characterize the symptoms of this form of hepatitis in primates by studying clinical, biochemical, histopathological and virological parameters; (2) To obtain a sufficient amount of PBMC, liver and lymphoid tissue for laboratory investigations, aimed at characterization of the infectious agent.

30

##### 2. Materials and methods

###### 2.1 Animals

Animals for the transmission studies were secured through the Biomedical Primate Research Center (BPRC) in Rijswijk, the Netherlands. Animal care and experimental conditions were in accordance with protocols that met all relevant requirements for the human care and the ethical use of primates in an approved facility. One female chimpanzee (*Pan troglodytes*) and four rhesus monkeys (*Macaca mulatta*), which were selected for the study, met the following criteria: (1) They had not been inoculated before with human serum or serum derived products. (2) They were negative for antibodies against hepatitis C virus and the core protein of hepatitis B virus. Hepatitis C and G virus RNA could not be detected and the tuberculin reaction was negative. (3) They showed normal levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase, and lactate dehydrogenase (LDH), which were determined at least 8 times in a 4-8 weeks interval before the inoculation.

(4) All animals showed normal liver histology with the absence of liver immunoreactivity using the monoclonal antibody HCV.OT 1F.

## 2.2 Transmission studies

### 2.2.1 Primary inoculations

Four different inocula were used in the first transmission study and were derived from two patients suspected of infection with a non A-G hepatitis inducing agent. The first patient (OHN) is a woman of 35 years old with a history of a blood transfusion in 1983. In august 1995 she complained of tiredness and was found to have a dramatic rise in serum ALT (8-10 times the upper normal limit), persisting for six months at the time of the liver biopsy. The liver biopsy showed a marked steato-hepatitis, with a granular staining for the monoclonal antibody HCV.OT 1F. Anti-HCV, serum HCV-RNA and HGV-RNA, and other known viral and autoimmune markers were repeatedly negative. There was no evidence for metabolic liver pathology. Plasma and peripheral blood mononuclear cells (PBMC) were with her permission stored at -80°C for further study. The second patient (CD) is a man of 39 years old who presented with recurrent oesophageal variceal bleeding as a complication of cirrhosis in December 1995. The cirrhosis could not be attributed to any known viral, toxic or metabolic cause, except for a positive immunoreactivity to HCV.OT 1F. In march 1996 he underwent an orthotopic liver transplantation. The explanted liver showed a micronodular type of cirrhosis,

with a pronounced mononuclear cell infiltrate in the portal tracts and a positive immunoreactivity using the monoclonal antibody HCV.OT 1F. The left liver lobe of the explant was used for isolation of hepatocytes by a two-step perfusion technique as described previously (Moshage H et al., 1988). The isolated primary human hepatocytes were subsequently cryopreserved. In addition, pre-transplantation plasma was also stored at -80°C.

Inoculum A contained 20 million cryopreserved human hepatocytes from patient CD, 2 ml plasma from patient CD, 5 ml plasma and PBMC (out of 20 ml blood) from patient OHN. Inoculum B was similar to inoculum A, but included no PBMC. Inoculum C contained only 20 million cryopreserved hepatocytes from patient CD, while inoculum D was prepared from 5 ml plasma of patient OHN.

Inoculum A was injected intravenously to chimpanzee Sylvia. Inocula B, C and D were injected to the rhesus monkeys I FU, 8925 and LXX respectively. The rhesus monkeys were monitored clinically and biochemically at least every two weeks for six months. Liver biopsy was performed monthly. The chimpanzee was followed in a similar but extended protocol and had an additional liver biopsy 18 months after inoculation.

### 2.2.2 Passage of 1 FU infectivity

Rhesus monkey I FU developed hepatitis, which was associated with an impressive liver immunoreactivity for the monoclonal antibody HCV.OT 1F (as described below in 3. *results*). Serum collected during the early acute phase (from 4 to 12 weeks after inoculation) was pooled (inoculum E) and injected to rhesus monkey BB58. This primate was monitored clinically, biochemically and histologically for 6 months.

### 2.3 Immunohistochemistry

Immunohistochemistry was performed as described previously (Yap SH et al., 1994) on 4 µm-thick cryostat sections of fresh frozen materials using a three-step indirect immunoperoxidase procedure.

Sections were incubated overnight at 4 °C with the monoclonal antibody HCV.OT 1F (purified IgG 20 ng/µl). The secondary and tertiary antibodies consisted of peroxidase-conjugated rabbit anti-mouse and peroxidase -conjugated swine anti-rabbit IgG, respectively

(both obtained from Dakopats a/s Copenhagen, Denmark; working dilution 1/50 and 1/100, respectively). Each incubation was performed for 30 minutes at room temperature and followed by a wash in three changes of phosphate buffered saline, pH 7.2. The reaction product was developed by incubation for 15 minutes in 100 mM acetate buffer (pH 5.2), containing 0.05 % 3-amino-9-ethyl-carbazole and 0.01 % H<sub>2</sub>O<sub>2</sub>, resulting in bright red staining of immuno-reactive sites. In order to abolish a weak "non-specific" background reactivity, the monoclonal antibody was pre-incubated overnight with the homogenate of normal human liver tissue before use.

#### 2.4 Assay for liver enzymes

Serum activities of aminotransferases (ALT and AST), alkaline phosphatase and gamma-glutamyltransferase (GGT) were determined using routine laboratory procedures. Baseline serum levels in international units per liter (IU/L) were established on serum specimens obtained weekly or bi-weekly. A minimum of eight values were obtained prior to inoculation. Cut-off values were determined for each animal, based on the mean enzyme value plus 3.75 times the standard deviation. Enzyme values above the cut-off value were interpreted as abnormal and suggestive of liver damage, as has been performed by others in similar transmission studies (Schlauder G et al., 1995).

### 3. Results

#### 3.1 Primary inoculations

##### 3.1.1 Chimpanzee Sylvia

From week 5 to 7 after inoculation there was a transient increase in serum AST above the baseline cutoff value, and a discrete rise in serum ALT as shown in figure 2. A liver biopsy performed 3 weeks after inoculation showed an increase in mononuclear cells in the parenchyma (lobular hepatitis) which was maintained during the observation period of 6 months. In addition, there was some degree of portal and periportal inflammation at week 7, 11 and 13. In addition, the biopsy taken at the time of the peak AST value (week 7) showed a moderate degree of piecemeal necrosis. This biochemical and histological hepatitis was associated with a granular immunoreactivity to HCV.OT 1F, in the form of cytoplasmatic



granules in the periphery of the liver cells which remained present up to 18 months after inoculation, with the exception of a negative staining at six months.

### 3.1.2 Rhesus monkey IFU

In contrast to the chimpanzee data there was no rise in serum liver enzymes following the inoculation throughout 6 months of observation (figure 3). However, the liver biopsy taken 1 month after inoculation showed a discrete lobular hepatitis with a few cells showing a positive immunoreactivity to HCV.OT 1F. At week 8 there was a moderately dense infiltrate in the portal tracts and borderline interphase hepatitis, associated with an intense HCV.OT 1F immunoreactivity. The subsequent biopsies showed a virtually normal liver parenchyma and a negative HCV.OT 1F immunoreactivity, with the exception of a few positive cells in the liver specimen at 20 weeks. Unfortunately, the final liver biopsy at six months was complicated by a hemorrhage and the primate died.

### 3.1.3 Rhesus monkey 8925

The inoculation of 20 million putatively infected hepatocytes resulted in a rise of serum ALT above the baseline cutoff value at week 11, and from week 16 to 17 (figure 4). The first liver biopsy at 4 weeks after inoculation was normal, including a negative HCV.OT 1F immunoreactivity. However, the staining became positive on the subsequent biopsy at 8 weeks and remained weakly positive for 6 months. At 10 weeks there was a distinct lobular hepatitis, which resolved on the subsequent biopsies. Eight months after inoculation the animal was sacrificed and the hepatocytes were subsequently isolated and cryopreserved for further study. However, at that time the liver was found to have lost immunoreactivity to HCV.OT 1F.

### 3.1.4 Rhesus monkey IXK

Inoculation with 5 ml plasma of patient OHN did not result in biochemical or histological hepatitis and there was no immunoreactivity to HCV.OT 1F.

### 3.2 Passage of IFU infectivity

Rhesus monkey BB58 did not have a rise in serum aminotransferases. However, two months after inoculation a focus of necrotising hepatitis was seen in the liver specimen and at 3 months a transient typical granular immunoreactivity to HCV.OT 1F was seen in a minority of the liver cells.

## 4 Discussion

From this transmission studies, both in chimpanzee and rhesus monkeys, we have provided solid evidence that the HCV.OT 1F immunoreactivity is caused by a transmissible agent, present in the hepatocytes of a patient (CD), who developed cirrhosis, characterized by liver immunoreactivity to HCV.OT 1F, despite negative anti-HCV or serum HCV-RNA. Moreover, this immunoreactivity to HCV.OT 1F was associated with a transient rise in serum ALT and a persistent inflammation in the liver parenchyma (both lobular and portal) in the chimpanzee study. The rhesus monkey I FU inoculated with the mixed inoculum, derived from the liver and plasma of patient CD and plasma of patient OHN, also developed this hepatitis, which resolved after 6 months. In addition, the development of transient liver immunoreactivity to HCV.OT 1F in rhesus monkey 8925, who was only inoculated with the hepatocytes of patient CD, further underscores the transmissibility of the putative non-A-G agent. The data of the passage study of I FU infectivity (rhesus monkey BB58) suggest that the agent can be transmitted by the serum of a primate with liver immunoreactivity to HCV.OT 1F. The lack of liver immunoreactivity in the primate LXX, who was inoculated only with the plasma of patient OHN, therefore suggests that the putative agent was not present in a sufficiently high titer in that plasma batch.

In conclusion, primary inoculation studies have shown that liver HCV.OT 1F immunoreactivity and associated hepatitis can be passed on a chimpanzee or rhesus monkeys by infusion of hepatocytes from a patient with non-A-G. In addition, liver HCV.OT 1F immunoreactivity could be transmitted from one rhesus monkey to another by infusion of serum, collected during the episode of hepatitis, which developed in a rhesus monkey after primary inoculation with a human putative non-A-G inducing agent.

## References

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**EXAMPLE 5**

**Characterization of an *in-vitro* assay using an immortalized human hepatocyte cell line for demonstration of hepatitis Y infection**

**1. Definition**

In this report we summarize the characterization of an *in-vitro* assay of HY infection, which is based on the incubation of immortalized human hepatocytes with inoculum containing non-A-G transmissible agent(s) in a rotatory cell culture system.

**2. Materials and methods****2.1 Development and characterization of the cell line**

The immortalized human hepatocyte line C (deposited at the ECACC under accession number 98121503) has been developed in our laboratory from primary cultures of human hepatocytes as described previously (Fourneau I et al., 1997). In brief, human hepatocytes were isolated from human liver tissues, which could not be used for transplantation for some reasons. These liver tissues were obtained from post mortem liver donors with approval of the

medical Ethical Committee for human experimentations. Hepatocytes were isolated using a two-step collagenase perfusion method (Moshage H et al., 1988; Rijntjes PJM et al., 1986). Freshly isolated hepatocytes were cultured on six well plates (Nunc, Roskilde, Denmark), pretreated with human liver biomatrix (Rijntjes PJM et al., 1988) and were allowed to attach to this matrix in complete WE medium (William's E medium, (Gibco) supplemented with 10% fetal calf serum (Gibco), 2mM L-Glutamine (Gibco) 20 mU/ml insulin (Novo Nordisk Pharma) 50 nm dexamethason and antibiotics).

The isolated hepatocytes were kept in culture for two months or longer. During this period of culture, cells were regularly investigated for spontaneously developing colonies using phase-contrast microscopy. When such colonies were observed, they were passaged to a new well using Trypsin-EDTA (Gibco, Bethesda, MD, USA). Only those growing colonies of cells, which were able to secrete human serum albumin, were further passaged and by this way we have established cell line C, in addition to a number of other cell lines. After trypsinization the immortalized human hepatocytes were resuspended in medium containing 10 % DMSO and 20 % fetal calf serum and stored in liquid nitrogen. Each cryovial contains 5 x 10<sup>6</sup> cells.

TABLE 6

	Cell line C	Primary human hepatocytes
<b>Cytokeratin expression (% of cells)</b>		
• CK7	-	-
• CK8	50	60
• CK18	100	95
<b>Bilirubin conjugation (<math>\mu\text{mol}/\mu\text{g DNA}/24 \text{ h}</math>)</b>		
• Total	0.47	0.79
• Mono-conjugate	0.23	0.55
• Di-conjugate	0.24	0.24
• Ratio di-/mono	1.04	0.44

**Protein secretion**  
( $\mu\text{g}/\mu\text{g DNA}/24 \text{ h}$ )

5	• Human serum albumin	70	120
	• Fibrinogen (minus IL-6)	4.1	310
10	• Fibrinogen (plus IL-6)	41	not done

Table 6 : Cytokeratin expression, protein secretion and bilirubin conjugation of the immortalized human hepatocyte line C in comparison with freshly isolated human hepatocytes.

15 As shown in table 6 the pattern of cytokeratin (CK) expression of cell line C reveals a low level of CK7 and a high level of CK8 and CK18 expression, which are characteristic for adult human hepatocytes. Cell line C secretes human serum albumin and the base-line fibrinogen production can be enhanced by interleukin-6 (IL-6) treatment. In addition, the ability to conjugate bilirubin is comparable with that of primary human hepatocytes. Finally,  
20 this C cell line was not found to be immunoreactive for the monoclonal antibody HCV.OT 1F.

## 2.2 Cell culture system

### 2.2.1 Culture of immortalized human hepatocytes (C-cell line) starting from cryopreserved cells

25 The following protocol is designed for the culture of immortalized hepatocytes (cell line C) starting from one cryovial containing 5 million hepatocytes.

2.2.1.1 Thaw one cryovial on ice;

2.2.1.2 Resuspend the cells in ice cold 10 ml of William's E (WE) medium (Gibco, Bethesda, MD, USA) at 4°C in a conical tube (Sarstedt, Numbrecht,

30 Germany);

2.2.1.3 Centrifuge for 5 min, at 800 rpm (143 xg) and 4°C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.1.4 Discard the supernatant;

2.2.1.5 Resuspend the cells carefully in 10 ml of WE-medium;

2.2.1.6 Centrifuge again for 5 min, at 800 rpm (143 g) and 4°C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.1.7 Discard the supernatant;

2.2.1.8 Resuspend the cells carefully in 1 ml of WE-medium supplemented with 10 %  
 5 Fetal Calf Serum (Gibco, Bethesda, MD, USA), 2 mM L- glutamine (Gibco), 20 mU/ml insulin (Novo Nordisk Pharma), 50 nM dexamethason, 50 µg/ml gentamycin (Schering-Plough), 100 µg/ml vancomycin (Lilly), 2.5 µg/ml fungizone (Bristol-Meyers Squibb) and 100 U/ml penicillin (Continental Pharma) (complete WE-medium);

2.2.1.9 Transfer the cell suspension to a 25 cm<sup>2</sup> culture flask (Nunc, Life

10 Technologies, Roskilde, Denmark) and 5 ml complete WE medium is added.

2.2.1.10 Culture cells at 37 °C and 5% CO<sub>2</sub>;

2.2.1.11 Change medium every 3 days;

2.2.1.12 Evaluate cell confluency by phase contrast microscopy on a  
 day to day basis;

15 2.2.1.13 When 90 to 100 % cell confluency has been reached, cells should be trypsinised.

#### 2.2.2 Trypsinisation of monolayer cell cultures (C-cell line)

2.2.2.1 Discard medium from culture flasks;

2.2.2.2 Wash cells 1 time with Phosphate Buffered Saline (PBS);

20 2.2.2.3 Add 5 ml Trypsin-EDTA (Gibco, Bethesda, MD, USA) to a 25 cm<sup>2</sup> flask or 10 ml to a 75 cm<sup>2</sup> flask;

2.2.2.4 Incubate at 37 °C, 5 % CO<sub>2</sub> for 5 minutes;

2.2.2.5 Detach the cells from the flask by shaking;

2.2.2.6 Add two volumes of cold WE-medium (4 °C) to stop the trypsin activity;

25 2.2.2.7 Centrifuge the cells for 5 min at 800 rpm and 4°C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.2.8. Discard supernatant;

2.2.2.9. Resuspend the cell pellet in complete WE-medium.

30 2.2.3 *Culture of immortalized human hepatocytes (C-cell line) in a Rotating Cell Culture System*

The immortalized human hepatocytes are cultured in a Rotating Cell Culture System (RCCS, Synthecon, Houston, USA), which is a vertically rotated, culture vessel with a membrane that allows gas exchange. The cells establish a uniform, low shear, fluid suspension orbit within the vertically rotating culture vessel. In this microgravity system, the absence of damaging stress forces allows three dimensional aggregation of cells (Schwarz RP et al., 1992).

A 50 ml volume RCCS vessel has a 1/2 inch fill port and two smaller syringe ports. Cells are cultured in the RCCS according to the manufacturer's instructions :

2.2.3.1 Transfer the RCCS vessel to a Laminar Flow Hood. Remove the end caps and place them on sterile petri dishes;

2.2.3.2 Wash the vessel with PBS;

2.2.3.3 After trypsinisation (2.2.2.9) dilute the hepatocyte suspension (C-cell line) in complete WE-medium to yield a final concentration of  $0.5 \times 10^6$  cells per ml;

2.2.3.4 Load the cell suspension into the RCCS-vessel through the 1/2 inch port;

2.2.3.5 An empty 5 ml sterile syringe is attached to one of the syringe ports. The syringe valve is opened and gently tap on sides to expel air bubbles from under the port. Close the syringe port.

2.2.3.6 Attach the vessel to the rotator base in a CO<sub>2</sub> (5%)-incubator, at 37°C;

2.2.3.7 Turn on power and adjust initial rotation speed of 15 to 20 rpm;

2.2.3.8 As cell aggregates grow, the rotation speed is adjusted to compensate for increased sedimentation rates.

#### 2.2.4 *Change medium and harvesting of cell aggregates*

2.2.4.1 Turn off power and remove the vessel from the rotator base and take it to a sterile hood;

2.2.4.2 Open the 1/2 inch fill port and remove cell suspension by aspiration with a 25 ml pipet;

2.2.4.3 Centrifuge the cell suspension for 5 minutes at 800 rpm and 4 °C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.4.4 Discard supernatant;

2.2.4.5 Take cell aggregates with a sterile glass pipet from the cell pellet. For immunocytochemistry (see 2.5) immerse the cell aggregates in liquid nitrogen chilled 2-Methylbutane (Sigma-Aldrich, Steinheim, Germany) and store at -20 °C;

5 2.2.4.6 Resuspend the remaining cell pellet in complete WE-medium. Load the cell Suspension into the RCCS-vessel and proceed according to the protocol discussed above (2.2.3.5 - 2.2.3.8).

### 2.3 Source and preparation of the inoculum

10 The inoculum was derived from the liver of patient CD, who underwent an orthotopic liver transplantation (OLT) for end stage chronic liver disease, most probably due to chronic infection with non-A-G-agent. Patient CD is a man of 39 years old who presented with recurrent oesophageal variceal bleeding as a complication of portal hypertension due to cirrhosis in december 1995. The cirrhosis could not be attributed to any known viral, toxic or  
15 metabolic cause, except for a positive immunoreactivity to HCV.OT 1F on a liver biopsy. In march 1996 he underwent an orthotopic livertransplantation. The explanted liver showed a micronodular type of cirrhosis, with pronounced mononuclear cell infiltrates in the portal tracts and the immunoreactivity to monoclonal antibody HCV.OT 1F was confirmed. Since all serological markers of known viral infections were repeatedly negative, including serum anti-  
20 HCV and HCV-RNA, this patient was considered of having HY.

#### 2.3.1 Cryopreserved non-A-G infected primary human hepatocytes

The left lobe from the explanted liver was used for isolation of the hepatocytes by a method as described above, which were subsequently cryopreserved (inoculum A).

#### 2.3.2 Homogenate of cryopreserved NON-A-G infected primary human hepatocytes

25 Ten million cryopreserved human hepatocytes were homogenized with a Dounce homogenizer, which yielded inoculum B. Inoculum B was separated in a supernatant (inoculum C) and pelleted fraction (inoculum D) by centrifugation (250 x g for 10 minutes, 4°C).

#### 2.3.3 Homogenate of NON-A-G infected liver tissue

30 Liver tissue from this patient, which was deep frozen in liquid nitrogen immediately after OLT, was homogenized and fractionated, according to the following protocol:



2.3.3.1 Thaw 2.0 gram of frozen liver tissue on ice in 20 ml ice-cold buffer TENB (0.05 M Tris, 0.001 M EDTA, 0.1 M NaCl; pH 7.5);

2.3.3.2 Homogenize sequentially by the Polytron (5 x 1 minute) and 4 times by a Dounce homogenizer;

5 2.3.3.3 Centrifuge the homogenate at 250 g for 10 min (4°C) and discard the pellet;

2.3.3.4 Centrifuge the supernatant from the previous step at 12,000 x g for 20 min (4°C) and discard the pellet;

2.3.3.5 Dilute the supernatant 6 x in TENB

2.3.3.6 Centrifuge the solution at 251,800 x g for 2 hours (20°C).

10 2.3.3.7 Dilute the supernatant from step 2.3.3.6 10 x in complete WE medium (inoculum E);

2.3.3.8 Resuspend the pellet from step 2.3.3.6 in 15 ml of TENB. Inoculum F consisted of 5 ml of this solution.

## 15 2.4 Incubation of the immortalized human hepatocytes with inoculum in the RCCS

The immortalized human hepatocytes were cultured in 6 RCCS vessels (25 x 10E6 cells/vessel) and incubated with the different NON-A-G inocula (A-F):

20 2.4.1 Inoculum A : 10 x 10E6 cryopreserved NON-A-G infected primary human hepatocytes (see 2.3.1);

2.4.2 Inoculum B : total homogenate of 10 x 10E6 cryopreserved NON-A-G infected primary human hepatocytes (see 2.3.2);

2.4.3 Inoculum C : supernatant fraction of inoculum B (after centrifugation for 10 minutes, at 250 x g) (see 2.3.2);

25 2.4.4 Inoculum D : pelleted fraction of inoculum B (after centrifugation for 10 minutes, at 250 x g) (see 2.3.2);

2.4.5 Inoculum E : supernatant fraction of homogenized NON-A-G infected liver tissue (see 2.3.3.7);

30 2.4.6 Inoculum F : pelleted fraction of homogenized NON-A-G infected liver tissue (see 2.3.3.8).

Medium was changed and cell aggregates were harvested every 3 to 4 days, starting at day 2 after infection.

## 2.5 Immunocytochemistry

Immunocytochemistry was performed as described previously (Yap SH et al., 1994) using a three-step indirect immuno-peroxidase procedure:

- 5        2.5.1    Cut sections (4  $\mu$ m thick) of fresh frozen materials and place on slides as for routine histological examination
- 2.5.2    Fix in acetone for 10 minutes at room temperature. Then air dry;
- 2.5.3    Rinse with distilled water and place in phosphate buffered saline (PBS) pH 7.2 for 5 minutes
- 10       2.5.4    Incubate for 30 minutes with the mouse monoclonal antibody HCV.OT 1F (purified IgG<sub>1</sub> : 50 ng / $\mu$ l). The monoclonal antibody was pre-incubated overnight with the homogenate of normal human liver tissue before use
- 2.5.5    Tap off antibody and place slide in PBS bath for 5 minutes;
- 2.5.6    Incubate for 30 minutes with peroxidase conjugated rabbit anti-mouse
- 15    immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:50 in PBS;
- 2.5.7    Tap off antibody and place slide in PBS bath for 5 minutes;
- 2.5.8    Incubate for 30 minutes with peroxidase conjugated swine anti-rabbit immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:100 in PBS;
- 2.5.9    Tap off antibody and place slide in PBS bath for 5 minutes;
- 20       2.5.10    Incubate for 10 minutes in 100 mM acetate buffer (pH 5.2), containing 0.05 % 3-amino-9-ethyl-carbazole and 0.01 % H<sub>2</sub>O<sub>2</sub>;
- 2.5.11    Rinse with distilled water;
- 2.5.12    Counterstain and mount with coverslip.
- 2.5.13    Evaluate microscopically for bright red granular staining of immunoreactive
- 25    sites in the cytoplasm of the hepatocytes.

## 3. Results

- 3.1 Co-culture of the C-cell line with NON-A-G infected cryopreserved primary
- 30    hepatocytes (inoculum A)

Two days after initiation of the co-culture some immuno-reactivity to HCV.OT 1F was seen in a minority of viable cells. However, eight days later this HCV.OT 1F

immunoreactivity was even more pronounced. The latter finding suggested that the immortalized human hepatocytes acquired HCV.OT 1F immunoreactivity when they were co-cultured with HCV.OT 1F immunoreactive primary hepatocytes. However, in order to exclude that the HCV.OT 1F immunoreactive cells were surviving primary hepatocytes and not  
5 immortalized hepatocytes, additional experiments with incubation of homogenized tissue or cells were performed.

3.2 Incubation of the C-cell line with total (inoculum B) and fractionated (inoculum C and D) homogenate of NON-A-G infected cryopreserved primary hepatocytes

10

Four days after start of the incubation of the C-cell line with the total homogenate (inoculum B) a typical granular immunoreactivity for HCV.OT 1F was seen in a minority of cells, which increased at day 8 and 12. The staining remained positive until day 24, after which the HCV.OT 1F immunoreactivity was lost.

15

At day 8 after infection with the supernatant fraction (inoculum C) of the cell homogenate a transient granular positivity was observed in the harvested hepatocytes, which was lost at the subsequent time points. However, at day 29 and 33 some positivity was observed again in a minority of cells. After infection with the pelleted fraction (inoculum D) of the cell homogenate no positivity for HCV.OT 1F could be seen until day 47 after infection,  
20 which adds to the specificity of the observed staining in the parallel experiments.

3.3 Incubation of the C-cell line with fractionated homogenate of NON-A-G infected liver tissue (inoculum F and F)

25

Twelve days after incubation with the supernatant fraction of the liver homogenate (inoculum F) a marked HCV.OT 1F immunoreactivity was observed, which was maintained at least until day 24 (last sample evaluated).

30

After incubation with the pelleted fraction (inoculum F) it took 21 days for some cells to gain HCV.OT 1F immunoreactivity, which was less pronounced than the cells in the parallel experiment. It should be noted that the viability of the cells was less after incubation with inoculum F, which may have been too concentrated or toxic for the cells.



•Yap SH, Willems M, Van Den Oord A et al.: Detection of HCV antigen by immunohistochemical staining : a histological marker of HCV infection. *J Hepatol* 1994; 20: 275-281.

5        **EXAMPLE 6**

**ISOLATION AND CHARACTERIZATION OF POLYNUCLEOTIDE SEQUENCES OF THE ETIOLOGICAL AGENT OF HEPATITIS Y**

10        Previous experiments revealed that a transmissible agent might be contained in sera or plasma or liver of patients with non-A-G hepatitis (see above). As a working hypothesis we assumed that such agent might be a virus and provisionally termed it Hepatitis Y virus (HYV). In order to isolate nucleic acid from HYV the following experiment was conducted.

15        **Representational Difference Analysis (RDA)**

**Overview of the method**

20        The protocol begins with the isolation of the total nucleic acid of the tester samples (presumed HepY positive material) and the driver samples (Hep Y negative material). The isolation was performed using the Boom method. The total nucleic acid content was converted into cDNA using random hexamers as non-specific primers. The resulting cDNA was cut by the restriction enzyme Sau3A I, which has a recognition site of four nucleotides ( GATC ). Using these Sau3A I sites adapters were ligated onto the cDNA molecules. PCR reactions  
25        were performed with adapter primers resulting in 'representations', of both tester- and driver-material. The tester representation was restricted by Sau3A I to remove the adapters and new adapters with a different sequence were ligated onto this representation. Tester and driver material were mixed, a hybridisation reaction was performed, followed by a PCR reaction with the same adapter primers as ligated to the tester representation. Therefore only tester-  
30        tester hybrids were presumed to be amplified exponentially by these primers. The material obtained after the first subtractive hybridisation was called Difference Product 1 (DP1). Adapters were removed from the amplified testermaterial by Sau3A I digestion and different

adapters were ligated to the testermaterial only. By changing the adapter primers of the testermaterial a couple of times, it was possible to perform a number of subtractive hybridisation's, thereby enriching tester specific sequences. Finally, the remaining tester material was isolated and purified from gel, cloned into a pGem4Z-vector by using the Sau3A I sites, and sequenced. These sequences were compared with different sequence databases. Sequences that remain unknown were used to design PCR and/or NASBA primers. These sequences were tested on negative and positive control genomic DNA samples and those sequences with no genomic DNA homologues were further investigated.

#### Example 1: Detailed protocol

##### 1) Tester preparation

Tester samples: - patient OHN serum December 1996

- patient OHN serum August 1997
- Macaque 1FU serum 21/1/97
- Chimp Sylvia plasma 4/2/97

(The patient and the animals, from which these samples were obtained, were at least once positive by immunostaining of liver sections with the HCV.OT 1F monoclonal antibody)

Total nucleic acid from 1 ml serum was isolated using the "Boom" method ( Boom R et al, 1990; J Clin Microbiol.28: 495-503 ) with 9 ml of lysisbuffer (pH 6.2, 4.7 M Guanidine isothiocyanate, Triton X-100, Tris/HCl)

After elution in 100 µl DEP-C-treated water( Rnase, Dnase free, Ambion) the nucleic acid was precipitated overnight at a temperature of -20 °C, by adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample was centrifuged during one hour at 25,000 rpm at 4 °C ( 120,000xg; Beckman Sw 28 rotor).

After air-drying, the pellet was dissolved in a mixture of 0.2 µl RNase inhibitor (40 units/ul) and 4.8 µl DEP-C-treated water.

## 2) Driver preparation

Driver sample: - Livertissue L701

(This sample was obtained from a human liver not reacting with the monoclonal antibody HCV.OT 1F)

5

A few slices of liver (about 10 mg) were isolated using the "Boom" method with 9 ml of lysisbuffer (4.5 M Guanidine isothiocyanate, pH 6.2).

The total nucleic acid content was eluted in 100 µl DEPC-treated water.

10

## 3) First strand synthesis

To the 5 µl tester or driver sample 0.7 µl of DEPC-treated water and 0.3 µl of random hexamers (50 ng/µl) were added.

15

This mixture was incubated during 10 minutes at 70 °C and next during 1 minute at 4 °C.

The following was added: - 1 µl of 10 X RTbuffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl)

- 1 µl of a 25 mM MgCl<sub>2</sub> solution

- 0.5 µl of a 10 mM dNTP solution

20

- 1 µl of 0.1 M DTT.

This mixture was incubated during 5 minutes at a temperature of 25 °C and then 0.5 µl of Superscript II enzyme (200 units/µl, RNase H negative reverse transcriptase , Gibco BRL) was added.

Incubation conditions:

25

- 10 minutes at 25 °C.

- 50 minutes at 42 °C.

- 15 minutes at 70 °C.

30

## 4) Second-strand synthesis

Immediately after the first strand reaction the following reagents were added to the reaction mixture:

To the 1  $\mu$ l sample the following reagents were added:



- 0.8 µl adapter mix (containing 0.5 µg of R-Bgl-12 and 0.25 µg of R-Bgl-24)
- 0.5 µl of DNA dilution buffer (5X)
- 2.5 µl of T4 DNA ligase buffer (2X)
- 0.3 µl of T4 DNA ligase

5 Incubation conditions:

15 minutes at room temperature

1 hour at 16 °C.

The sequences of the adapters were:

R-Bgl-12 5'- GATCTGCGGTGA-3'

10 R-Bgl-24 5'- AGCACTCTCCAGCCTCTCACCGCA-3'

The following product is formed:

5' -AGCACTCTCCAGCCTCTCACCGCAGATCXXXXXXXXXX-R-Bgl-12 -3'  
15 3' - AGTGGCGTCTAGYYYYYYYYYCTAG-R-Bgl-24 -5'

where XXXXX resp. YYYYYY are the target sequence and its complement.

### 7) Generation of the representations

20 For each sample, four 100  $\mu$ l PCR reactions were performed.

The products of the ligation reactions were diluted ten times in DEPC-treated water (input material).

For each PCR reaction the following reagents were mixed:

- 25
- 79.5 µl of DEPC-treated water
  - 10 µl of 10X PCR buffer (Perkin Elmer, 100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01% (w/v) gelatine)
  - 8 µl of a 2.5 mM dNTP-solution
  - 1 µl of R-Bgl-24 adaptor-primer (1 µg/µl)
  - 0.5 µl of input material

30 The sequence of the adaptor-primer was:

5'-AGCACTCTCCAGCCTCTCACCGCA-3'

This mixture was incubated for 3 minutes at a temperature of 72 °C and then to each reaction 1 µl of Amplitaq ( 5U/µl , Amplitaq DNA Polymerase, recombinant, thermostable, 94 kDa DNA Polymerase, Perkin Elmer) was added.

Incubation conditions:

- 5       - 5 minutes at 72 °C.
- 30 cycles:    1 minute; 95 °C.  
                          3 minutes; 72 °C.
- 10 minutes at 72 °C.

After these PCR reactions the representations were analysed on a 1.25% agarose gel.

10     DNA yield was estimated by using the extinction at 260 nm( OD260).

#### 8) Removing of adapter sequences

Both tester and driver representations were digested with Sau3A I to remove the adapters.

15       Ten µg of the tester representations was digested with 16 units Sau3A I in a total volume of 40 µl ( see par.5) and hundred µg of the driver representation was digested with 64 units Sau3A I in a total volume of 200 µl.

The digestion's were performed overnight at a temperature of 37 °C.

20       The digested driver representation was purified by a phenol/chloroform extraction, followed by an overnight precipitation at -20 °C., after adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample was centrifuged for 15 minutes at 13000 rpm () at 4 °C. After drying the pellet was dissolved in 50 µl of DEPC-treated water, DNA yield was calculated to be 1.058 µg/µl by using the OD260 value.

25

#### 9) Purification of the tester representation

Of the tester representations 200-600 bp fragments were isolated from an 1.5% agarose gel and purified by silica binding in the presence of high salt using the Qiagen Qiaex II Agarose Gel Extraction kit.

30       The samples were eluted in 40 µl of DEP-C-treated water.

The Qiaex eluates were precipitated by adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol, followed by an overnight incubation at a temperature of -20 °C.

5 The samples were centrifuged for 15 minutes at a speed of 13,000 rpm (Heraeus Sepatech table centrifuge) at 4 °C.

After drying the pellets, they were dissolved in 1 µl of DEP-C-treated water.

#### 10) Ligation of new adapters to tester material

For the adaptor ligation the rapid ligation kit of Boehringer Mannheim was used ( see  
10 also par.6).

The following reagents were mixed:

- 1 µl of 'Qiaex cleaned' tester DNA ( see par.9)
- 2.4 µl of 0.8 µl J adapter mix, containing 0.5 µg of J-Bgl-12 and 0.25 µg of J-Bgl24
- 1 µl of 5X DNA dilution buffer
- 15 - 5 µl of 2X DNA ligase buffer
- 0.6 µl of T4 DNA ligase

Incubation conditions:

15 minutes at room temperature

1 hour at 16 °C.

20 The sequences of the adapters were:

J-Bgl-12 5'-GATCTGTTCATG-3'

J-Bgl-24 5'-ACCGACGTCGACTATCCATGAACA-3'

After ligation the samples were diluted to a concentration of about 10 ng/µl.

#### 25 11) Subtractive hybridisation

As driver material in the hybridisation reaction the following mixture was used:

- 8.5 µg of cDNA from liver L701, isolated in step 8.
- 11.5 µg of Human Cot-1 DNA (Gibco BRL)

As tester material 400 ng of adaptor ligated tester material was used, leading to a  
30 proportion of driver and tester in this hybridisation of 50:1 (w/w).

The driver and tester material was combined and 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol were added.

Precipitation: - 1 hour at - 70 °C.

- 5 minutes at 37 °C.

The samples were centrifuged for 15 minutes at 13000 rpm() and 4 °C.

After drying the pellets, they were dissolved in 4 µl 3XEE buffer (30 mM EPPS, pH 8.0;

5 3 mM EDTA)

Incubation conditions:

: - 5 minutes at 37 °C.

- 5 minutes at 98 °C.

After reducing the temperature to 67 °C., 1 µl of 5M NaCl was added.

10 This mixture was incubated for 20 hours at a temperature of 67 °C. Afterwards 395 µl of DEPC-treated water was added and the sample was stored at a temperature of -70 °C.

#### 12) Generation of first difference products (DP1)

For each sample, four 100 µl PCR-reactions were performed as described below:

15 - 60 µl of DEPC-treated water

- 10 µl of 10X PCR buffer (Perkin Elmer, 100 mM Tris-HCl, pH8.3; 500 mM

KCl; 15 mM MgCl<sub>2</sub>; 0.01% (w/v) gelatine)

- 8 µl of 2.5 mM dNTP-solution

- 20 µl of subtractive hybridisation product

20 This mixture was incubated for 3 minutes at 72 °C, 1 µl of Perkin Elmer Amplitaq (5 U/µl) was added, followed by an incubation for 5 minutes at 72 °C.

Then 1 µl J-Bgl-24 adaptor primer (1 µg/µl) was added.

Incubation conditions:

- 10 cycles 1 minute 94 °C.

25 3 minutes 70 °C.

- 10 minutes 72 °C.

After pooling the four reactions, the samples were purified by performing a phenol/chloroform extraction.

The samples were precipitated for 1 hour at a temperature of -70 °C., after adding 0.1  
30 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The samples were centrifuged for 15 minutes at 13000 rpm at 4 °C.

After drying the pellets they were dissolved in 20 µl of DEPC-treated water.

To remove ssDNA molecules a treatment with mung bean nuclease was performed.

To each reaction the following reagents were added:

- 15 µl of DEPC-treated water

- 4 µl of 10X mung bean nuclease buffer 100 mM NaOAc, (pH 5.0), 1mM

5 ZnOAc, 10 mM L-cysteine, 500 mM NaCl, 50 % glycerol.

- 1 µl of mung bean nuclease (34 units/µl)

This mixture was incubated for 35 minutes at a temperature of 30 °C and then 160 µl of 50 mM Tris-HCl pH 8.9 was added.

This was incubated for 5 minutes at 98 °C.

10 For the secondary PCR-reactions (four reactions for each sample) the following reagents were mixed:

- 60 µl of DEPC-treated water

- 10 µl of 10X PCR buffer (Perkin Elmer, 100 mM Tris-HCl, pH8.3; 500 mM

KCl; 15 mM MgCl<sub>2</sub>; 0.01% (w/v) gelatine)

15 - 8 µl of 2.5 mM dNTP solution

- 1 µl J-Bgl-adaptor-primer (1 µg/µl)

To this 20 µl of the mung bean nuclease treated product was added and the mixture was incubated for 1 minute at 95 °C.

After cooling the mix to 80 °C. 1 µl of Amplitaq (PE, 5 units/µl)) was added.

20 Incubation conditions of the following PCR:

: - 20 cycles 95 °C., 1 minute

70 °C., 3 minutes

-final cycle 72 °C., 10 minutes

After the PCR the reactions were pooled and 10 µl was analysed on a 1.5% agarose gel; 25 the remainder was purified by a phenol/chloroform extraction.

After addition of 0.1 volume of 3M NaOAc (pH5.3) and 2.5 volumes of ice-cold 96% ethanol, the samples were precipitated overnight at -20 °C.

After centrifugation at 13000 rpm (at room temperature the pellets were airdried and dissolved in 50 µl DEPC-treated water.

30 DNA yield was calculated using the OD260 values.

### 13) Generation of DP2

For the generation of DP2 the same protocol (steps 8 -12) was used as described for the generation of DP1. After replacing the tester adaptor with the N-Bgl adaptor, a new subtractive hybridisation was performed.

The N-adaptor sequences are:

5 N-Bgl-12 5'-GATCTTCCCTCG-3'

N-Bgl-24 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

The proportion driver:tester was 100:1.

As tester material 250 ng of the ligated DP1-material was used and as driver material a mixture of 6.5 µg cDNA from liver L701 and 18.5 µg of human Cot-1 DNA. For the PCR  
10 with the N-Bgl-24 primer an annealing temperature of 72°C was used.

#### 14) Generation of DP3 (SB98018)

For the generation of DP3 the same principle was used as described for the generation of DP1. After replacing the N-Bgl tester adaptor with J-Bgl adaptor, a new subtractive  
15 hybridisation was performed.

For the generation of DP3 the driver:tester proportion was 800:1

As testermaterial 3.1 ng of the ligated DP2-material and as drivermaterial a mixture of 7 µg liver cDNA from liver L701 and 18 µg of human Cot-1 DNA was used. For the PCR with the J-Bgl-24 primer an annealing temperature of 70°C was used.

20

#### 15) Analysis of the DP3-products

Bands of interest in the DP3 products were isolated from a 1.5% agarose gel ( in 1xTAE buffer) and purified using the Qiaex II gel extraction kit (Qiagen).

A volume of 0.5 ul of these extracts was inserted in the pGEM T-vector (Promega).  
25 After ligation, the ligation products are 10x diluted in water and electroporated in MC1061 electrocompetent E.Coli cells. Products are plated on LB agar plates with Ampicillin (500:1). Plates are incubated overnight at 37°C. The insert colonies were screened on insert length with SP6 and T7 primers. The plasmids containing inserts were sequenced with SP6 and T7 promoter sequences as primers.

30 The inserts between two consecutive Sau3A I sites were investigated separately. The sequences presumed to be derived from genomic sequences not related to hepatitis Y were discarded. The following criteria were used:

- >90% homology of the insert to known sequences **or**  
- positive reaction in PCR using primers derived from the insert sequence on human genomic DNA yielding fragments having a length comparable to the PCR product of the plasmid containing the insert.

5 Of the hundred sequences analysed two sequences, both derived from OHN serum did not fulfil one of these criteria and were further analysed.

- sequence nd1.1b (derived from OHN serum December 1996)

- sequence nd2.1 (derived from OHN serum August 1997)

10 **EXAMPLE 7**

**Utility of RT-PCR using nested primer set and probe derived from SEQ ID-1**

**PROTOCOL RT-PCR**

15 ***RNA extraction.***

RNA was extracted from 200 µl serum or EDTA treated plasma, in a single step acid guanidinium thiocyanate-phenol-chloroform extraction procedure, as described by Chomczynski & Sacchi (*Anal Biochem* 1987; 162:1 56-159). After isopropanol precipitation, the RNA was dissolved in 8µl DEPC-treated water.

20

***HYV detection by RT-PCR assay.***

For reverse transcription, the RNA was denatured by incubation for 10 min at 60° C. To the RNA fraction, 12µl reverse-transcription mix was added, containing 200 units Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco BRL, Bethesda, MD), 30  
25 units placental RNase inhibitor (Pharmacia, Uppsala, Sweden), 80 pmol random primers (Boehringer Mannheim, Germany), 0.5 mM of each deoxynucleotide triphosphate (dNTP; Pharmacia), 10 mM dithiothreitol, an appropriate volume of 5 x reverse transcriptase buffer, and DEPC treated water. The cDNA synthesis was carried out at 37°C for 1 hour and stopped by heating in boiling water for 1 minute. Subsequently, double round nested PCR was  
30 performed. Briefly, the first PCR mixture contained 5 µl cDNA solution, 16 pmol of each HYV (first round) primer, 0.2 mM of each dNTP and 1 unit Primezyme DNA polymerase (Biometra, Gottingen, Germany) in a total volume of 50 µl PCR buffer (10 mM Tris-HCl pH

8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.01% gelatin). Samples were overlaid with mineral oil and subjected to thermal cycling. For the nested PCR, 5 µl of first round PCR product was added to a new PCR mixture containing the appropriate nested PCR primers for a second amplification. First and second round cycling conditions were 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, 35 cycles. Amplified DNA was visualized by agarose gel electrophoresis (2%) and ethidium bromide staining.

## RESULTS

We analysed the inocula used in the bioassay studies. Supernatant fraction S3 which resulted in a positive HCV.OT 1F staining in the immortalized hepatocytes was tested for the presence of sequence SEQ ID-1 by nested PCR. In a parallel experiment, a pellet of liver homogenate (P2) was also investigated, which was shown to give negative results in the bioassay studies.

- Supernatant fraction S3: +/+  
- Pellet fraction P2 -

### 2. Detection of sequence SEQ ID-1 in the patient population:

-hepatitis non A-G infected patients: 7/10 were found positive for sequence SEQ ID-1

Y1 : OHN plasma 7/97	+ / + / - / -
OHN serum 3/97	+
OHN serum 12/96	-
Y2: CD 12/95	+ / +
Y3: VG 8/97	+
Y4: RG 9/98	+ / -
Y5: CJ 3/96	+
Y6 : AL 10/95	-
Y7: ML 4/95	-
Y8: BW	- / -



WO 00/37650

85

PCT/EP99/10179

Y9 : CM 3/98 +

Y10 MG 1/96 +

negative control patients (HCV.OT 1F negative): 0/7 positive for sequence SEQ ID-1

5

N1 : DGu (toxic hepatitis) 8/97 -/-

N2: DGr (toxic hepatitis) 4/96 -

N3: LRe (toxic hepatitis) 5/95 -

N4: HCV.OT 1F negative donorliver -/-

10

N5 :VP (HBV infected) -

N6: HH (HBV infected) -

N7: LRi (HBV infected) 4/98 -

3. Detection of sequence SEQ ID-1 in healthy blood donors:

15

- 2 of 28 blood donors tested positive for sequence SEQ ID-1

- BD 44(274-6) en BD 53 (274-15): +

**EXAMPLE 8**

20

**Cesium chloride gradient ultracentrifugation fractions monitored by nested RT-PCR for HYV related sequence SEQ ID-1****Materials and methods**

25

## • Buffer G:

10 mM Hepes pH 7.4

75 mM KCl

5 mM MgCl<sub>2</sub>

500 mM NaCl

30

• Gradient solutions: 26 % CsCl<sub>2</sub> and 58 % CsCl<sub>2</sub> in buffer G

• Autoclave cesium chloride solutions

• Preparation of gradient in autoclaved RNase free centrifugation tubes

30      Overview of the protocol

For this approach the Clontech Marathon TM cDNA Amplification Kit is used. This is a method for performing both 5' and 3' rapid amplification of cDNA ends from the same template.

The viral RNA is converted into ds cDNA , primed by a random hexamer and the ds cDNA is blunt-ended ligated to the Marathon cDNA Adaptor. Next, this uncloned library of adaptor-ligated ds cDNA is used as input material to perform 5'- and 3' RACE PCR reactions. These PCR reactions are performed with an internal gene specific primer (GSP) and the adaptor primer (AP1).

The RACE products may be characterised on an ethidiumbromide-stained agarose gel. Bands of interest are isolated from gel, purified and cloned into a suitable vector by blunt-end ligation. Alternatively one of the specific cutting sites in the adaptor sequence may be used.

As input material for the RACE reactions the following cesium-chloride gradient fractions, as described in example 8, may be used:

Gradient IV (S3-supernatants) - fraction G-IV-7 (density 1.311g/ml)

- fraction G-IV-11 (density 1.226 g/ml)

Gradient V (OHN plasma 7/97) - fraction G-V-1 (density 1.359g/ml)

- fraction G-V-2 (density 1.356 g/ml)

- fraction G-V-4 (density 1.334 g/ml)

- fraction G-V-5 (density 1.337 g/ml)

- fraction G-V-11 (density 1.225 g/ml)

- fraction G-V-12 (density 1.207 g/ml)

#### Detailed protocol

##### \* Sample preparation

The samples are isopropanol precipitated at a temperature of -20 °C.

Samples are spun down in a table centrifuge during 30 minutes by 14000 rpm and a temperature of 4 °C.

Pellets are washed with cold 70% ethanol, followed by 5 minutes centrifugation by 14000 rpm and a temperature of 4 °C.

Pellets are dried during 5 minutes in a vacuum-drier and then redissolved in 25 µl of a solution of DEPC-treated water and a scent of RNA guard (Amersham-Pharmacia Biotech ).

When the pellets are redissolved well, they are pooled and a phenol/chloroform extraction is performed.

After the extraction, 1/10 volume of 3M NaAc (pH 5.3), 2 µl of PELLET PAINT™ co-precipitant(coloured glycogen)(Novagen) and 2.5 volumes of 96% ethanol (cold) is added  
5 and the sample is precipitated overnight at a temperature of -20 °C.

Samples are spun down in the ultracentrifuge during 1 hour at a speed of 25,000 rpm and a temperature of 4 °C.

After removing the supernatant, the pellet is dried during 10 minutes at roomtemperature and redissolved in 5 µl DEPC-treated water.

10

\* First-strand synthesis

To the 5 µl eluate 0.7 µl of DEPC-treated water and 0.3 µl of random hexamer (50 ng/µl) is added.

Incubation: - 10 minutes at 70 °C

15

- 1 minute at 4 °C.

Next the following is added: - 1 µl of 10 X PCR buffer (200 mM Tris-HCl (pH 8.4),  
500 mM KCl )

- 1 µl of a 25 mM MgCl<sub>2</sub> solution

- 0.5 µl of a 10 mM dNTP solution

20

- 1 µl of 0.1 M DTT.

This mixture is incubated during 5 minutes at a temperature of 25 °C and then  
0.5 µl of Superscript II enzyme (Gibco/BRL) is added.

Incubation: - 10 minutes at 25 °C.

- 50 minutes at 42 °C.

25

- 15 minutes at 70 °C.

After this incubation the reaction is stored on ice and followed immediately by the second-strand synthesis.

\* Second-strand synthesis

30

To the first-strand synthesis mixture (10 µl in total) the following is added:

- 48.4 µl of sterile, deionized water

- 16 µl of second strand buffer

- 1.5 µl of a 10 mM dNTP-mixture
- 4 µl of second-strand enzyme cocktail (*E. coli* DNA polymerase I (150 U),  
*E. coli* DNA ligase (30 U) and *E. coli* RNase H (6.25 U))

This is incubated during 90 minutes at a temperature of 16 °C And then 2 µl (10 units) of

5 T4 DNA polymerase is added.

Incubation is for 45 minutes at a temperature of 16 °C.

The reaction is purified by a phenol/chloroform extraction.

An overnight precipitation (-20 °C) is performed by adding 0.1 volumes of 3M NaAc (pH 5.3), 0.2 µl (2 µg) of tRNA carrier and 2.5 volumes of ice-cold 96% ethanol.

10 The sample is spundown in the ultracentrifuge during 1 hour at 25000 rpm and a temperature of 4 °C.

The pellet is air-dried during 10 minutes and redissolved in 5 µl of DEPC-treated water.

\* Adaptor ligation

15

To the 5 µl of cDNA the following is added:

- 2 µl of Marathon TM cDNA adaptor (10 uM)
- 2 µl of 5 X DNA ligase buffer (250 mM Tris-HCl (pH 7.8), 50 mM MgCl<sub>2</sub>, 5 mM DTT, 5 mM ATP, 25 % w/v Polyethyleneglycol Mw 8,000)

20 - 1 µl (400 units) of T4 DNA ligase (Promega)

After mixing and spinning down, the ligation-reaction is carried out overnight at a temperature of 16 °C.

Next the reaction is incubated at a temperature of 70 °C for 5 minutes to inactivate the T4 DNA ligase.

25 The reaction is stored at a temperature of -20 °C.

\* Amplification of cDNA Ends (RACE)

For each PCR reaction the following reagents are mixed:

- 30
- 36 µl of water
  - 5 µl of 10 X cDNA PCR reaction buffer (200 mM Tris-HCl (pH 8.4),  
500 mM KCl)

- 1 µl of a 10 mM dNTP mix
- 1 µl of Advantage cDNA Polymerase mix (50 X)(Clontech)

For the 5' RACE reaction the following reagents are mixed:

- 43 µl of the above described mixture
- 1 µl of a 10 mM adaptor primer (AP1)
- 1 µl of a 10 mM antisense gene-specific primer (GSP1, p554)
- 5 µl of the adaptor-ligated cDNA.

For the 3' RACE reaction the following reagents are mixed:

- 43 µl of the above described mixture
- 1 µl of a 10 mM adaptor primer (AP1)
- 1 µl of a 10 mM sense gene-specific primer (GSP2, p553)
- 5 µl of the adaptor-ligated cDNA

The following PCR reactions are performed (in a hot-lid thermal cycler):

- 94 °C for 30 seconds

- 5 cycles: 94 °C 30 sec

72 °C 4 min

- 5 cycles: 94 °C 30 sec

70 °C 4 min

- 25 cycles: 94 °C 30 sec

68 °C 4 min

After performing the PCR reactions 5 µl of the PCR-product is analysed on a 1.2% agarose gel. Bands or smears of interest are cut out of gel.

If the primary PCR reaction fails to give enough product to analyse on an agarose gel, a secondary, nested PCR, can be performed using a nested adaptor primer (AP2) and a nested gene-specific primer (NGSP, for 5'RACE: p550, for 3'RACE: p549).

In this case the product of the primary PCR reaction is diluted 50 times in water.

Of this dilution 5 µl can be used as input. The same PCR scheme can be used as described above.

After performing this nested PCR, the samples are again analysed on a 1.2% agarose gel.

Bands or smears of interest are cut out of the gel.

\* Analysis of the RACE products

These products are purified by using the Qiagen Qiaex gel extraction kit).

The purified material is analysed on agarose gel.

5 The DNA is then cloned into a conventional vector by using one of the restriction sites in the marathon adaptor or in a T/A cloning vector.

After cloning, the inserts of the clones are sequenced using standard methods.

The obtained sequences are screened in sequence databases.

10 A number of the obtained sequences remain unknown and contain that part of the clone that was used to design the gene-specific primers; these are further analysed in PCR and/or in NASBA.

The sequences are used to design new gene-specific primers.

With these new gene-specific primers, new RACE reactions are performed, the resulting fragments cloned and analysed, until no further extending sequences are found.

15

**2. 'Modified RDA'**

Overview of the protocol

20 The protocol begins with the isolation of the total nucleic acid of a serum sample by using the Boom method. The RNA is converted into DNA by performing a cDNA synthesis reaction using a random hexamer as a non-specific primer. The obtained cDNA is cut by the restriction enzyme, for instance BamH1, used as an example here but other restriction enzyme can be chosen, to generate DNA-fragments with a BamH1-site on both ends. Using these  
25 BamH1 sites, partially double-stranded adaptors are ligated onto the cDNA molecules. PCR is performed using the adaptor-primer to amplify this cDNA non specifically. The products are ligated into the T/A vector (Promega) or after restriction with Bam H1 into a BamH1 restricted pGem vector (Promega) and PCR is performed with 2.1 specific primers to detect the presence of the original sequence. With a combination of a gene specific primer, either  
30 forward or reversed, and vector specific primers (both SP6 and T7) clone 2.1 containing sequences are specifically amplified by PCR from this cloned, non specifically amplified fraction. This fraction is cloned in a T/A vector (Promega) and the inserts of these clones are

analysed by PCR with 2.1 specific primers. If 2.1 containing sequences are underrepresented in this population a further round of enrichment can be performed by repeating the amplification with vector- and 2.1 specific primers as described above. The inserts of 2.1 containing inserts are sequenced. These sequences are screened in the databases and unknown sequences (containing the previously known part) can be analysed by PCR. Clones remaining negative on negative control samples and giving positive results on the positive control samples, i.e samples of proven HepY patients, are further analysed.

The protocol described above may be repeated with any restriction enzyme (e.g. Bgl II or EcoR 1). In this way different cDNA-fragments arise, which leads to the elucidation of another part of the HYV genome. By alternating between these two preparations most of the HYV genome is amenable for analysis.

Detailed protocol ( see also example 6 for details)

\* Sample preparation

Total nucleic acid from 1 ml OHN serum is isolated by the Boom method, using GuSCN and silica binding.

After elution, the nucleic acid is precipitated overnight at a temperature of -20 °C, by adding 0.1 volumes of 3M NaAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample is spundown in the ultracentrifuge during one hour at 25000 rpm and a temperature of 4 °C.

After air-drying, the pellet is dissolved in a mixture of 0.2 µl RNase inhibitor and 4.8 µl DEPC-treated water.

\* First strand synthesis

To the 5 µl eluate 0.7 µl of DEPC-treated water and 0.3 µl of random hexamer (50 ng/µl) is added.

This mixture is incubated during 10 minutes at 70 °C and next during 1 minute at 4 °C.

The following is added: - 1 µl of 10 X PCR buffer(200 mM Tris-HCl (pH 8.4),

500 mM KCl)

- 1 µl of a 25 mM MgCl<sub>2</sub> solution

- 0.5 µl of a 10 mM dNTP solution



- 1  $\mu$ l of 0.1 M DTT.

This mixture is incubated during 5 minutes at a temperature of 25 °C.

0.5  $\mu$ l of superscript II enzyme is added.

Incubation: - 10 minutes at 25 °C.

5 - 50 minutes at 42 °C.

- 15 minutes at 70 °C.

After this incubation the reaction is stored on ice and followed immediately by the second-strand synthesis.

10 \* Second-strand synthesis

The second-strand synthesis is performed according to the Boehringer Mannheim protocol.

To the 10  $\mu$ l first strand reaction, the following reagents are added:

15 - 8  $\mu$ l of 2.5 X second strand buffer

- 2  $\mu$ l of DNA polymerase I (13.6 units)

- 0.5  $\mu$ l of RNase H (1 unit)

Incubation: - 120 minutes at 14 °C.

- 10 minutes at 70 °C.

20 0.5  $\mu$ l of T4 DNA polymerase (4 units) is added.

This mixture is incubated at 37 °C. for 10 minutes.

The reaction is purified by a phenol/chloroform extraction.

An overnight precipitation (-20 °C) is performed after adding 0.1 volume of 3M NaAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

25 The sample is spun down in the ultracentrifuge during 1 hour at 25000 rpm and a temperature of 4 °C.

After drying, the pellet is redissolved in 15  $\mu$ l of DEPC-treated water.

\* BamH1 digestion

30

Digestion with the enzyme BamH1 is performed in a total volume of 20  $\mu$ l:

- 15  $\mu$ l of ds cDNA

- 4 µl of 10 X One PhorAll buffer (Promega)

- 1 µl of BamHI (10 units)

This reaction is incubated at a temperature of 37 °C. for 15 hours.

The reaction is purified by a phenol/chloroform extraction.

5 An overnight precipitation (-20 °C) is performed by adding 0.1 volumes of 3M NaAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample is spun down in the ultracentrifuge during 1 hour at a speed of 25000 rpm and a temperature of 4 °C.

After drying, the pellet is redissolved in 1 µl of DEPC-treated water.

10

\* Adaptor ligation

For the adaptor ligation we use the RapidLigation kit (Boehringer Mannheim)

To the 1 µl sample the following reagents are added:

- 15
- 0.8 µl adaptormix (containing 0.5 µg of 24-mer and 0.25 µg of 12-mer)
  - 0.5 µl of DNA dilution buffer (5X)
  - 2.5 µl of T4 DNA ligase buffer (2X)
  - 0.3 µl of T4 DNA ligase

Incubation: 15 minutes at roomtemperature

20 1 hour at 16 °C.

\* Nonspecific amplification of adaptor-ligated cDNA-molecules

For each PCR reaction the following reagents are mixed:

- 25
- 13.2 µl of DEPC-treated water
  - 2 µl of 10X PCR buffer (Perkin Elmer)
  - 1.6 µl of a 2.5 mM dNTP-solution
  - 1 µl of the 24 mer adaptor primer (1 µg/µl)
  - 1 µl of the ligation-product

30

  - 0.2 µl of Perkin Elmer amplitaq

The following cycle scheme is used (in a hot-lid thermal cycler):

- 94 °C. for 30 seconds

- 30 cycles: 94 °C. 30 seconds
- 70 °C. 4 minutes

After performing the PCR reactions 5 µl of the PCR-product is analysed on a 1.2% agarose gel, the remainder is cleaned by the Qiaex procedure and cloned directly into the T/A vector (Promega).

For this purpose the following ingredients were mixed:

	T-vector (50 ng/µl, Promega)	1 µl
	DNA ligase buffer, 10* :300mM Tris-HCl, pH 7.8	
	100 mM MgCl <sub>2</sub>	
10	100 mM DTT	
	10 mM ATP	
		1 µl
	insert	1 µl
	T4 DNA ligase (3units/µl, Promega)	0.5 µl
15	bidistilled water	6.5 µl

The ligation was performed overnight at 16°C.

#### Semi-nested amplification of adaptor-ligated cDNA-molecules

20 The cloned PCR preparation was subjected to a second round of PCR

For each PCR reaction the following reagents were mixed:

- 14 µl of aqua bidest
- 2 µl of 10X PCR buffer (Perkin Elmer) (200 mM Tris-HCl (pH 8.4), 500 mM KCl, Perkin Elmer)
- 25 - 1.6 µl of a 2.5 mM dNTP-solution
- 1 µl of the Sp6 promotor or T7 promotor specific primer (1 µg/µl)
- 1 µl of the gene-specific primer (P553 or P554; 1 µg/µl)
- 1 µl of the ligation-product
- 0.2 µl of Perkin Elmer Amplitaq

30 The following cycle scheme was used (in a hot-lid thermal cycler):

- 94 °C. for 30 seconds
- 30 cycles: 94 °C. 30 seconds

70 °C. 4 minutes

Sequentie P553: 5-CCCGACGAACGTACGCTGAGCGTA

Sequentie P554: 5-CGCTCGATGGCGTACCACAGCTC

5

After performing the PCR reactions 5 µl of the PCR-product was analysed on a 1.2% agarose gel. The products were purified using the Qiagen Qiaex protocol.

The DNA then was cloned into the T/A cloning vector (Promega).

For this purpose the following ingredients were mixed:

10	T-vector (50 ng/µl, Promega)	1 µl
	DNA ligase buffer, 10* 300mM Tris-HCl, pH 7.8	
	100 mM MgCl <sub>2</sub>	
	100 mM DTT	
	10 mM ATP	
15		1 µl
	insert	1 µl
	T4 DNA ligase (3units/µl, Promega)	0.5 µl
	bidistilled water	6.5 µl

The ligation was performed overnight at 16°C.

20 Electrocompetent TopF-cells (Invitrogen) were transformed with ten times diluted ligation mix with sterile water following standard procedures .

DNA of the plasmids containing 2.1 derived inserts, as deduced by PCR with SP6 and T7 primers succeeded by a PCR with 2.1 specific primers (P443 and P442), were sequenced.

25

Sequentie P442: 5-CACGCCCTCGACAAACAGCG

P443: 5-TCTGCACGCCTTGGTTTGCA

According to the above protocol, the sequences shown in SEQ ID NO's 17 to 26 were identified as partial clones derived from the Hepatitis Y genome.

## CLAIMS

1. A method for detecting non-A-G hepatitis virus in a sample negative for HCV wherein said sample is contacted with antibodies obtainable from a cell line deposited at the ECACC under accession number 9110711 or monoclonal antibodies having the same immunoreactivity whereafter the presence of immunocomplexes formed is detected and from this the presence of non-A\_G hepatitis virus is determined.
2. An immortalised human hepatocyte cell line deposited at the ECACC under accession number 98121503.
3. Use of the cell line according to claim 2 in the in vitro diagnosis of non-A-G hepatitis.
4. Hepatitis Y virus characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement.
5. Nucleic acid sequence derivable from the genome of Hepatitis Y virus or from fragments of said genome or functional equivalents of said nucleic acid sequence, wherein Hepatitis Y virus is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.
6. A polypeptide comprising an amino acid sequence or fragment thereof wherein said amino acid sequence is encoded by a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement, or any functional equivalents of said polypeptide.
7. Antibodies reactive with a polypeptide according to claim 3 or functional equivalents thereof.
8. Method for the detection of hepatitis Y virus in a sample comprising the steps of isolating nucleic acid from said sample and hybridising said nucleic acid with a nucleotide sequence derived from the nucleic acid sequence of claim 2.
9. Method for the detection of hepatitis Y virus in a sample according to claim 5 characterised in that said nucleotide sequence is derived from the sequence in SEQ ID NO: 1.

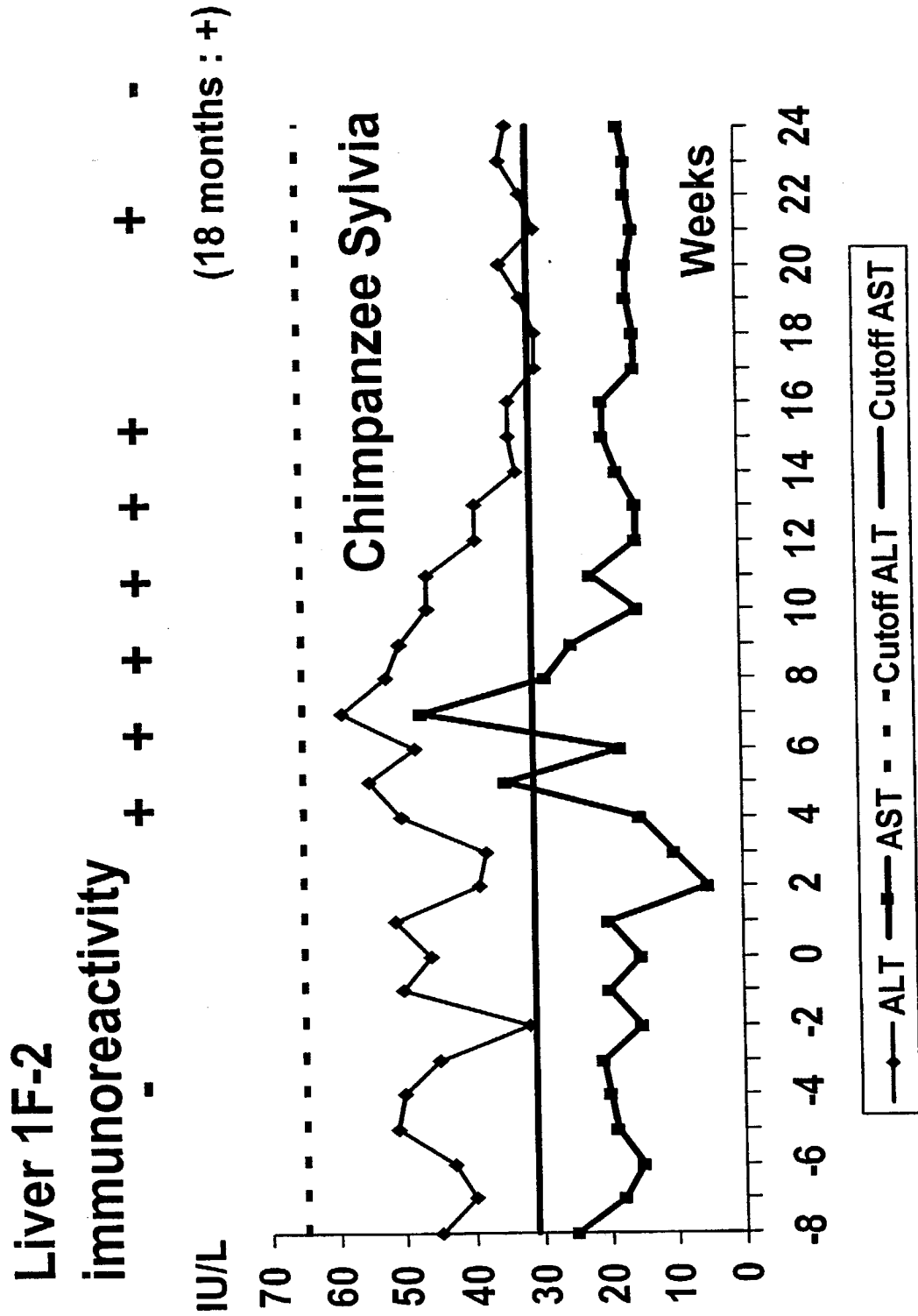
- 10      Method for diagnosing infection with hepatitis Y virus comprising the steps of  
5      providing a sample from a patient or animal suspected of being infected with  
Hepatitis Y virus, providing a hepatitis Y virus according to claim 1 or a  
polypeptide according to claim 3 and establishing immunoreactivity of said  
virus or polypeptide with antibodies in said sample.
- 11      Method for diagnosing infection with hepatitis Y virus comprising the steps of  
10      providing a sample from a patient or animal suspected of being infected with  
Hepatitis Y virus, providing an antibody according to claim 4 and establishing  
whether immunoreactive components are present in said sample.
- 12      Method for growing Hepatitis Y virus (HYV) comprising providing cells  
15      infected with HYV and propagating said cells in vitro, wherein HYV is  
characterised in that it comprises a genome comprising a nucleotide  
sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement  
or the sequence of SEQ ID NO: 2 or its complement.
- 13      A vaccine composition comprising a polypeptide according to claim 3 in  
20      substantially isolated form mixed with a pharmaceutically acceptable  
excipient
- 14      A vaccine composition comprising a nucleic acid sequence according to  
25      claim 2 in substantially isolated form mixed with a pharmaceutically  
acceptable excipient

# Figure 1



Figures 2/6

Figure 2

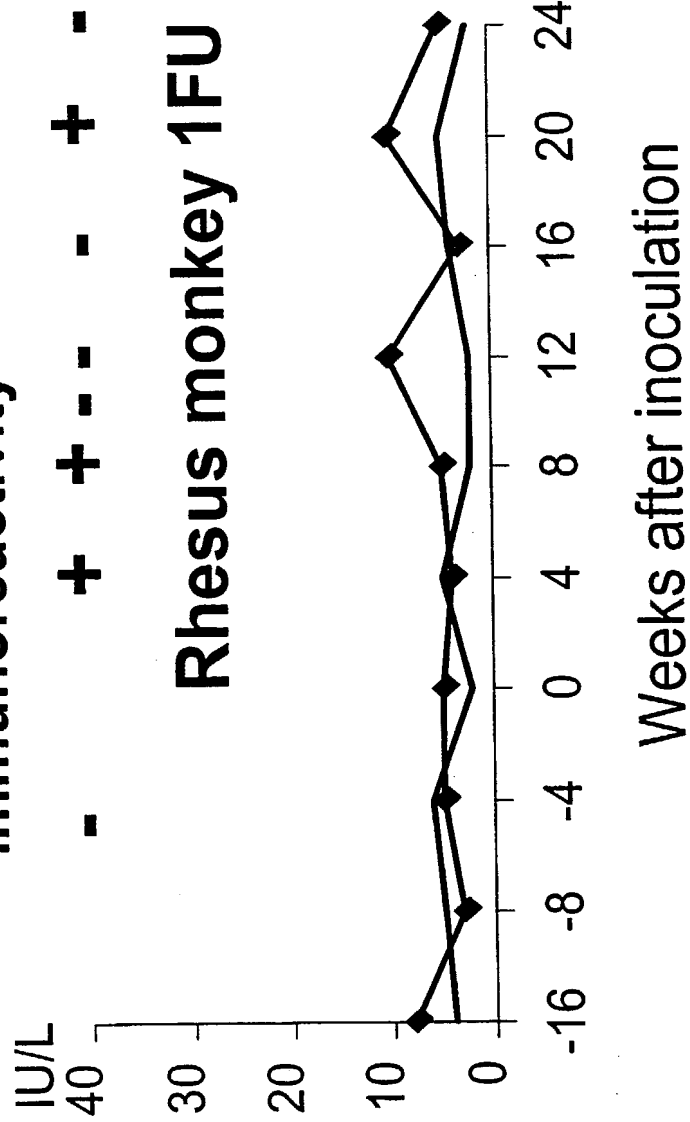




Figures 3/6

Figure 3

Liver HCV.OT 1F  
immunoreactivity



**Figures 4/6** **Figure 4** **Liver 1F-2** **Rhesus monkey 8925**

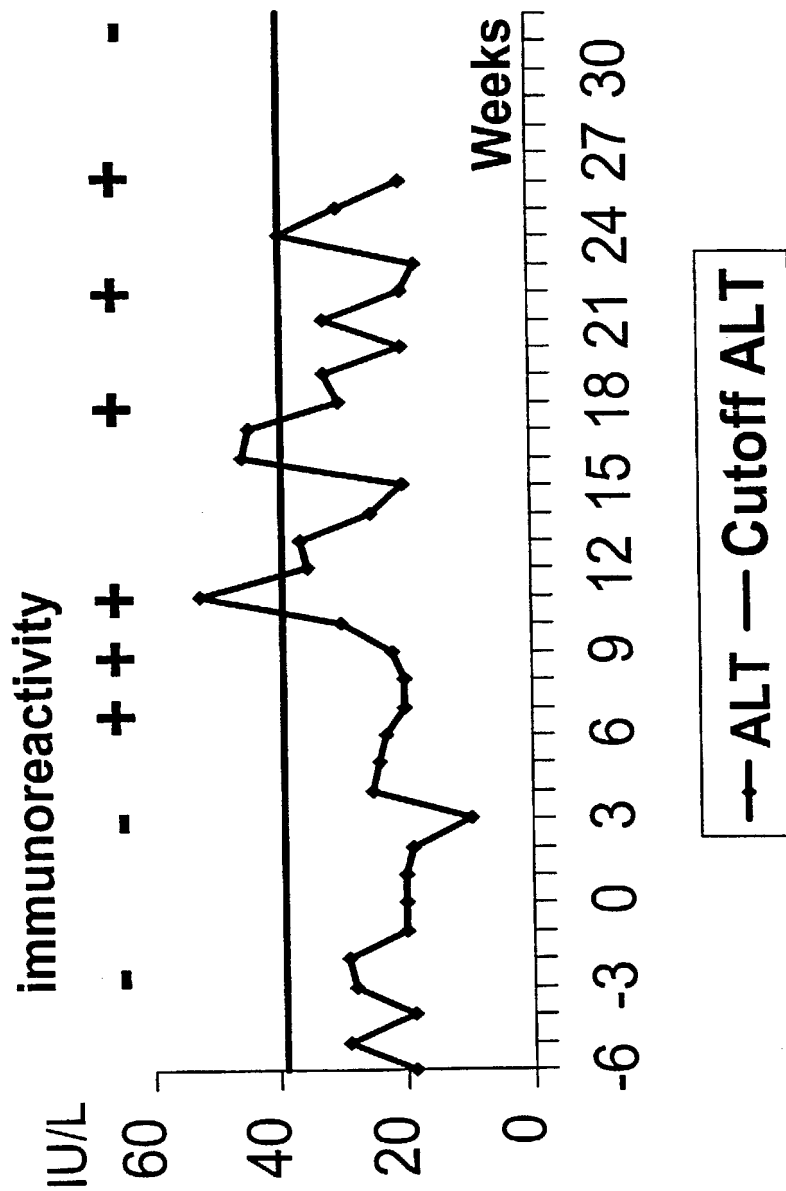
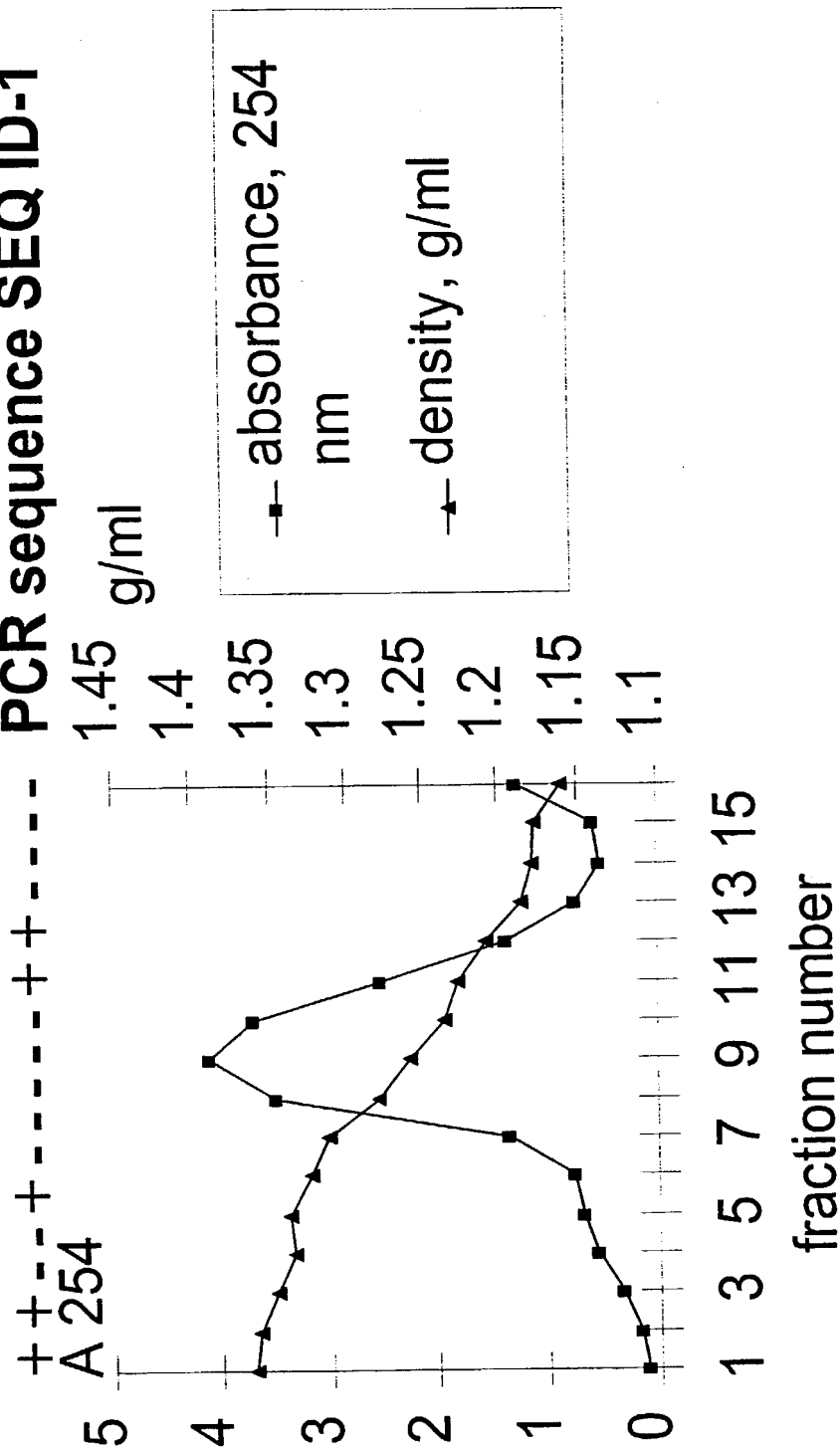


Figure 5

Figures 5/6

Cesium chloride gradient      Plasma of patient OHN

PCR sequence SEQ ID-1



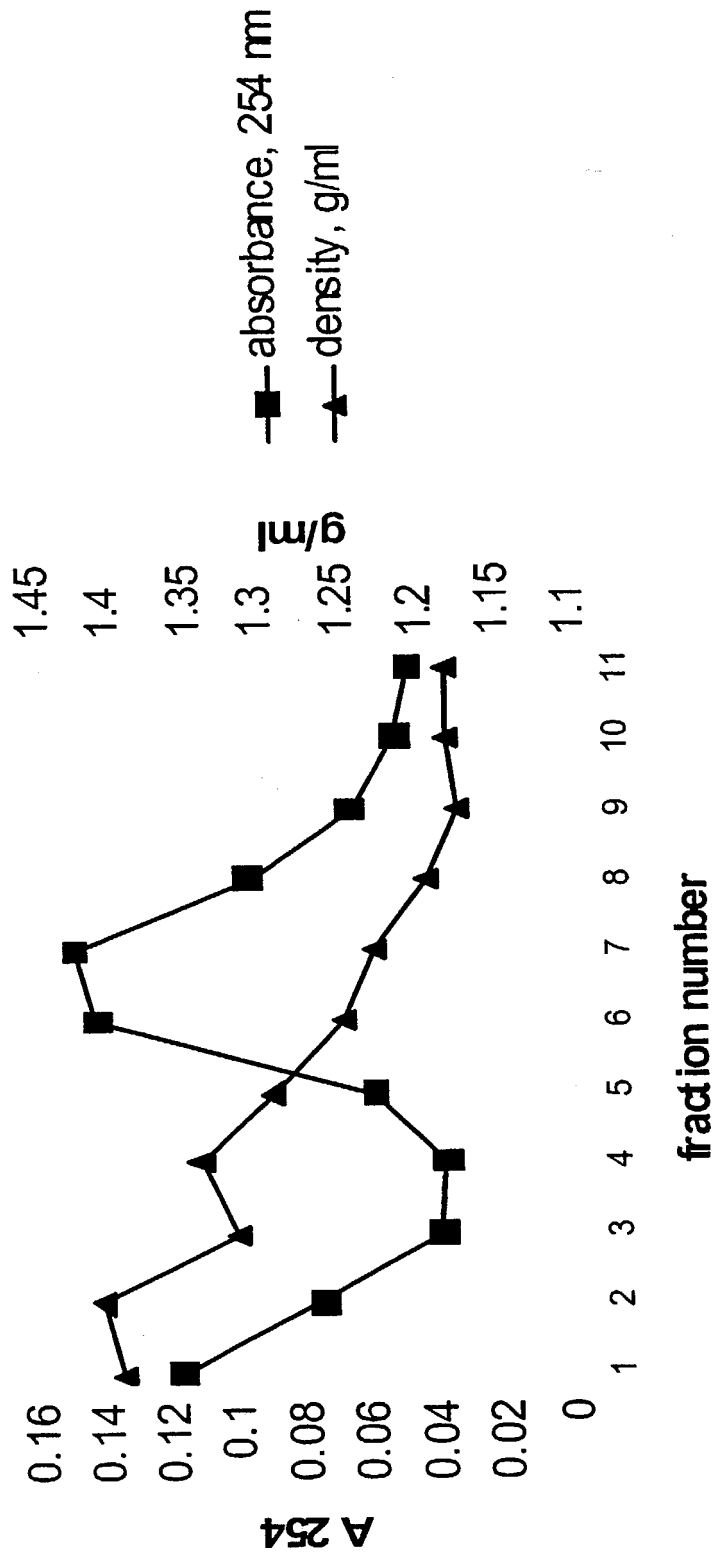
Figures 6/6

Figure 6

Cesium chloride gradient

Supernatant fraction S3 (liver of patient CD)

- - - - - + - - - - + PCR sequence SEQ ID-1



**DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**  
Attorney Docket No. \_\_\_\_\_

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled \_\_\_\_\_

the specification of which

☐ is attached hereto

OR

☒ was filed on 16 December 1999 as United States Application No. or PCT International Application Number PCT/EP99/10179 and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56, including material information that became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate, or of any PCT International application having a filing date before that of the application on which priority is claimed.

|            |         |                   |   |
|------------|---------|-------------------|---|
| 98204313.5 | EP      | December 18, 1998 | <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |
| Number     | Country | MM/DD/YYYY Filed  | Priority Claimed  |
| 99200167.7 | EP      | January 20, 1999  | <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |
| Number     | Country | MM/DD/YYYY Filed  | Priority Claimed  |
|            |         |                   | <input type="checkbox"/> Yes <input type="checkbox"/> No            |
| Number     | Country | MM/DD/YYYY Filed  | Priority Claimed  |

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

|                              |                                 |
|------------------------------|---------------------------------|
|                              |                                 |
| <b>Application Number(s)</b> | <b>Filing Date (MM/DD/YYYY)</b> |
|                              |                                 |
| <b>Application Number(s)</b> | <b>Filing Date (MM/DD/YYYY)</b> |

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or § 365(c) of any PCT international application designating the United States of America, listed below.

|                          |                    |  |
|--------------------------|--------------------|--|
|                          |                    |  |
| <b>Appln. Serial No.</b> | <b>Filing Date</b> | <b>Status<br/>Patented/Pending/Abandoned</b> |
|                          |                    |  |
| <b>Appln. Serial No.</b> | <b>Filing Date</b> | <b>Status<br/>Patented/Pending/Abandoned</b> |
|                          |                    |  |
| <b>Appln. Serial No.</b> | <b>Filing Date</b> | <b>Status<br/>Patented/Pending/Abandoned</b> |

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following registered attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. I also appoint the following registered attorney(s) to represent me before all competent International Authorities in connection with any and all international applications filed by me with an appropriate receiving office claiming priority to the U.S.

application. I also appoint the following registered attorney(s) to make or receive payment on my behalf in connection with the filing of such international applications.

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application. I also appoint the following registered attorney(s) to make or receive payment on my behalf in connection with the filing of such international applications.

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Attorney's Docket No. 9250-37

PATENT

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Yap et al.  
Serial No.: To be assigned  
Filed: Concurrently herewith  
For: HEPATITIS Y VIRUS

Date: June 18, 2001

Commissioner for Patents  
Washington, DC 20231

## ASSOCIATE POWER OF ATTORNEY

Sir:

The undersigned attorney of record in this case hereby appoints the below named persons, individually and collectively, as associate attorneys to prosecute this patent application and to transact all business in the United States Patent and Trademark Office connected therewith and the resulting patent:

## CUSTOMER NUMBER 20792

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| Sorojini J. Biswas    | 39,111 |
| Needham J. Boddie, II | 40,519 |
| James R. Cannon       | 35,839 |
| James D. Myers        | 28,330 |
| Julie H. Richardson   | 40,142 |
| Kenneth D. Sibley     | 31,665 |

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Raleigh, North Carolina 27627

In re: Yap et al.  
Serial No.: To be assigned  
Filed: Concurrently herewith

Please direct all telephone calls about this application and the resulting patent  
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The above-appointed associate attorneys are hereby authorized to act and rely  
on instructions from and communicate directly with the assignee of record and with  
such patent agent/attorney liaison, individual or firm, selected by the assignee, as may  
represent themselves to the associate attorneys as having been so appointed, unless  
and until I instruct the associate attorneys in writing to the contrary.



Frans GM Hermans

P.O. Box 20  
Weth. Van Eschstraat 1  
5340 BH Oss  
The Netherlands

Date: June 14, 2001

WO 00/37650

## SEQUENCE LISTING

PCT/EP99/10179

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5

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(H) TELEFAX: 0412 650592

10

15

(ii) TITLE OF INVENTION: Hepatitis Y Virus

(iii) NUMBER OF SEQUENCES: 26

## (iv) COMPUTER READABLE FORM:

20

- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 304 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40

GATCACAAGC AACTGCCCCGA CGAACGTACG CTGAGCGTAT TCGTCGACGA ACTGCACGCC 60  
CTCGACAAAC AGCGCCTGTC CGGCAAGCTG TCCGAGGAGT TCAACCGCGC CTATACCGGC 120  
45 ATGTCCAGCG TGGCCAAAGC CACTGCCCCG CGCGTTGGCC GACTGGACGC CCAGGCGCTG 180  
CAAAGCCAAG GCGTGCAGAC GCTGCTCGAG GCCCACC GCA ACTGGAGCAA GCCCGAGCTG 240  
TGGTACGCCA TCGAGCGCGC CGGCAAGGTT TACACCTACG ATTACTACCT GACCGCACTG 300

50

WO 00/37650

PCT/EP99/10179

GATC

304

## (2) INFORMATION FOR SEQ ID NO: 2:

- 5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 222 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATCGABGTG CAACACGCCC GCCTATHACG GCGCGTATTG CTTGTBGCAG CCTGAGTGCA 60

20

GCATTAGATT AGCCAATTAT CTGGGGCACC ATCATAAGCA GAAGGGATAA GCATGGCGCT 120

CACCGACCAA TCCACCCGCA CCCGTACCGG CGAAGAACTC GACGCTGCCG TCATCGACGC 180

CTATCTCAAG GCCCATATTC CCGGCCTGAG TGGCGAGGCC GG 222

25

## (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 15 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

40

CGTACGCTGA GCGTA

15

## (2) INFORMATION FOR SEQ ID NO: 4:

- 45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 15 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single

**WO 00/37650**

**PCT/EP99/10179**

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

10 GGCGTACCAC AGCTC

15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CACGCCCTCG ACAAACAGCG

20

(2) INFORMATION FOR SEQ ID NO: 6:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

35

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTGCACGCC TTGGTTTGCA

20

45

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTGCAACACG CCCGC

15

15

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

30

CCGGCCTCGC CACTC

15

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACGGCGCGTA TTGCTTGT

18



WO 00/37650

PCT/EP99/10179

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCCGGAATA TGGGCCTT

18

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ser Gln Ala Thr Ala Arg Arg Thr Tyr Ala Glu Arg Ile Arg Arg Arg  
 1 5 10 15

Thr Ala Arg Pro Arg Gln Thr Ala Pro Val Arg Gln Ala Val Arg Gly  
 20 25 30

Val Gln Pro Arg Leu Tyr Arg His Val Gln Arg Gly Gln Ser His Cys  
 35 40 45

Pro Ala Arg Trp Pro Thr Gly Arg Pro Gly Ala Ala Lys Pro Arg Arg  
 50 55 60

Ala Asp Ala Ala Arg Gly Pro Pro Gln Leu Glu Gln Ala Arg Ala Val  
 65 70 75 80

Val Arg His Arg Ala Arg Arg Gln Gly Leu His Leu Arg Leu Leu Pro  
85 90 95

5            Asp Arg Thr Gly  
                                 100

(2) INFORMATION FOR SEQ ID NO: 12:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 101 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

- (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

25      Ile Thr Ser Asn Cys Pro Thr Asn Val Arg Xaa Ala Tyr Ser Ser Thr  
          1                       5                       10                       15

Asn Cys Thr Pro Ser Thr Asn Ser Ala Cys Pro Ala Ser Cys Pro Arg  
20 25 30

30 Ser Ser Thr Ala Pro Ile Pro Ala Cys Pro Ala Trp Pro Lys Pro Leu  
35 40 45

Pro Gly Ala Leu Ala Asp Trp Thr Pro Arg Arg Cys Lys Ala Lys Ala  
35                    50                    55                    60

Cys Arg Arg Cys Ser Arg Pro Thr Ala Thr Gly Ala Ser Pro Ser Cys  
65 70 75 80

40 Gly Thr Pro Ser Ser Ala Pro Ala Arg Phe Thr Pro Thr Ile Thr Thr  
85 90 95

Xaa Pro His Trp Ile  
100

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 101 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

15

Asp His Lys Gln Leu Pro Asp Glu Arg Thr Leu Ser Val Phe Val Asp  
1 5 10 15

Glu Leu His Ala Leu Asp Lys Gln Arg Leu Ser Gly Lys Leu Ser Glu  
20 25 30

20

Glu Phe Asn Arg Ala Tyr Thr Gly Met Ser Ser Val Ala Lys Ala Thr  
35 40 45

25

Ala Arg Arg Val Gly Arg Leu Asp Ala Gln Ala Leu Gln Ser Gln Gly  
50 55 60

Val Gln Thr Leu Leu Glu Ala His Arg Asn Trp Ser Lys Pro Glu Leu  
65 70 75 80

30

Trp Tyr Ala Ile Glu Arg Ala Gly Lys Val Tyr Thr Tyr Asp Tyr Tyr  
85 90 95

Leu Thr Ala Leu Asp  
100

35

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 101 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: protein

45

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

[illegible]

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

30                   (A) LENGTH: 101 amino acids  
                    (B) TYPE: amino acid  
                    (C) STRANDEDNESS: single  
                    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

45

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ile | Gln | Cys | Gly | Gln | Val | Val | Ile | Val | Gly | Val | Asn | Leu | Ala | Gly | Ala |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |
| Leu | Asp | Gly | Val | Pro | Gln | Leu | Gly | Leu | Ala | Pro | Val | Ala | Val | Gly | Leu |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |

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Glu Gln Arg Leu His Ala Leu Ala Leu Gln Arg Leu Gly Val Gln Ser  
 35 40 45

Ala Asn Ala Pro Gly Ser Gly Phe Gly His Ala Gly His Ala Gly Ile  
 5 50 55 60

Gly Ala Val Glu Leu Leu Gly Gln Leu Ala Gly Gln Ala Leu Phe Val  
 65 70 75 80

10 Glu Gly Val Gln Phe Val Asp Glu Tyr Ala Gln Arg Thr Phe Val Gly  
 85 90 95

Gln Leu Leu Val Ile  
 100

15

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 100 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(v) FRAGMENT TYPE: internal

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Ser Ser Ala Val Arg Xaa Xaa Ser Xaa Val Xaa Thr Leu Pro Ala Arg  
 1 5 10 15

35

Ser Met Ala Tyr His Ser Ser Gly Leu Leu Gln Leu Arg Trp Ala Ser  
 20 25 30

40

Ser Ser Val Cys Thr Pro Trp Leu Cys Ser Ala Trp Ala Ser Ser Arg  
 35 40 45

Pro Thr Arg Arg Ala Val Ala Leu Ala Thr Leu Asp Met Pro Val Xaa  
 50 55 60

45

Ala Arg Leu Asn Ser Ser Asp Ser Leu Pro Asp Arg Arg Cys Leu Ser  
 65 70 75 80

Arg Ala Cys Ser Ser Ser Thr Asn Thr Leu Ser Val Arg Ser Ser Gly

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85

90

95

Ser Cys Leu Xaa  
100

5

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 376 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

20

|  |     |
|--|-----|
| CGCCCGGGCA GGTCTGTTCA TCCTCGTCAC CTTGTCGCC CCGATCGGTA CCATGCTGTG   | 60  |
| GCGCACGTGC ATCACCCGAC CGTGGCCGAG CTGATTCCCC TGACCCCTGGC CGAGCTCGAA | 120 |
| CGCTGGGATG ATCACAAGCA ACTGCCGGAC GAAAAAACCC TGCAGGTCTT CGCCAGCGAA  | 180 |
| CTGCATGGCC TTAACCAGCA GCGCCTGTCC GGCAAGCTCT CCGAAGAACT CAACCGCGCC  | 240 |
| TATACCGGCA TGTCCAGCGT GGTCAAAGCC ACTGCCCGGC GCGTTGGCCG ACTGGACGCC  | 300 |
| CAGGCGCTGC AAACCAAGGC GTGCGGACGC TGCTCGAGGC CCACCGCAAC TGGAGCAAGC  | 360 |
| CCGAGCTGTG GTACGC  | 376 |

30

35

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 378 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

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CGCCCCGGGCA GGTCTGTTCA TCCTCGTCAC CTTCGTCGCC CCGATCGGTA CCATGCTGTG 60  
 GCGCAGCGTG CATCACCCGA CCGTGGCCGA GCTGATTCCC CTGACCCTGG CCGAGCTCGA 120  
 5 ACGCTGGGAT GATCACAAGC AACTGCCGGA CGAAAAAACC CTGCAGGTCT TCGCCAGCGA 180  
 ACTGCATGGC CTTAACCAGC AGCGCCTGTC CGGCAAGCTC TCCGAAGAAC TCAACCGCGC 240  
 10 CTATACCGGC ATGTCCAGCG TGGTCAAAGC CACTGCCCCG CGCGTTGGCC GACTGGACGC 300  
 CCAGGCGCTG CAAAGCCAAG GCGTGCGGAC GCTGCTCGAG GCCCACCGCA ACTGGAGCAA 360  
 GCCCCGAGCTG TGGTACGC 378  
 15

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 404 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGAAGCGCG TAGCCCCATT TGTGTTCTGT GTGCCAATGC TGCTGTTCAT CCTCGTCACC 60  
 TTCGTCGCCC CGATCGGTAG GATCCTGTGG CGCAGCGTGC ATCACCCGAC CGTGGCCGAG 120  
 35 CTGATTCCCG TGACCCTGGC CGAGTCGAAC GCTGGGATGA TCACAAGCAA CTGCCGGACG 180  
 AAAAAACCCT GCAGGTCTTC GCCAGCGAAC TACNTNCCTT AACCAGCAGC GCCTGTCCGG 240  
 CAACTCTCCG AAGAACTCAA CCACGCCTAT ACCGGCATAT CCTGCGTGCT CAAATTTACT 300  
 40 GCCCCGGCGCG TTGGCCGACT GGACGCCAG GCGCTGCAAA GCCAAGGCGT GCAGACGCTG 360  
 CTCGAGGCCC ACCGCAACTG GAGCAAGCCC GAGCTGTGGT ACGC 404

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 366 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: unsure
- 10 (B) LOCATION: 363..366

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

|    |   |     |
|----|---|-----|
| 15 | CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCCTCGACAA ACAGCGCCTG TCCGGCAAGC | 60  |
|    | TGTCCGAGGA GTTCAACCGC GCCTATACCG GCATGTCCAG CGTGGTCAA GCCACTGCCC  | 120 |
|    | GGCGCGTTGG CCGACTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG ACGCTGCTCG | 180 |
| 20 | AGGCCCACCG CAACTGGAGC AAGCCCGAGC TGTGGTACGC CATCGAGCGC GCCGGCAAGG | 240 |
|    | TTTACACCTA CGATTACTAC CTGACCGGAC TGCATCTCGA GATCTATACT GACTAATCCC | 300 |
| 25 | TTGACCATCG CGACCAGAAG CATATCACCT CCCCGCCAAT TCGCAATATA CCTATANTTA | 360 |
|    | TANNNN  | 366 |

(2) INFORMATION FOR SEQ ID NO: 21:

- 30 (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 582 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - 35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

|    |   |     |
|----|---|-----|
| 45 | CACTTGTGGG AGGGGGGTTG GGGGAGAGGG TGAATCTGTC ACCATCTCCC CAGCCCTCTC | 60  |
|    | CGTAAACGGG AGAGGGAGCT ACTGATCGAC ACTTGCTGTG CGCCCTGTTT TACGGAGTAA | 120 |
|    | CACCTTGTC TCCCTGACCA CCAGCGAAGC CGGCCAAGCC GCCAGCGCCC GTCGCAAGAA  | 180 |



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5 GTGCGTCGCC GCCTTTCTGT TCGTGGTGCC ACTGCTGCTG TTCATCCTCG TCACCTTCGT 240  
CGCCCCGATC GGTACCATGC TGTGGCGCAG CGTGCAATCAC CCGACCGTGG CCGAGCTGAT 300  
TCCCCTGACC CTGGCCGAGC TCGAACGCTG GGATGATCAC AAGCAACTGC CGGACGAAAA 360  
AACCCTGCAG GTCTTCGCCA GCGAACTGCA TGGCCTTAAC CAGCAGCGCC TGTCCGGCAA 420  
10 GCTCTCCGAA GAACTCAACC GCGCCTATAC CGGCATGTCC AGCGTGGTCA AAGCCACTGC 480  
CCGGCGCGTT GGCCGACTGG ACGCCCAGGC GCTGCAAAGC CAAGGCGTGC AGACGCTGCT 540  
CGAGGNNCAC CGCAACTGGA GCAAGCCCGA GCTGTGGTAC GC 582

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 513 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

30 CCAATATGTT GGAGCTGCTT TGATATGTTC GACATATAGT CGACCTGAAT ACTGATGAAT 60  
TAGTATTTGG TTAGTGTGGC GGTCGGGGCA GGTCCAGCGC AGGTCGCAAG AAACGCCTCG 120  
35 CTGCCTTTCT CTTCGTGGTA CCGCTGCTGC TGTTCATCAT CGTCACCTTC GTCGCCCCGA 180  
TCGGTACCAT GCTGTGGCGC AGCGTGCATC ACCCGACCGT GGCCGAAGT ATTCTCTGA 240  
CCCTGGCCGA ACTCGAGCGC TGGGACGATC ACAAGCAACT GCCCGACGAA CGTACGCTGA 300  
40 GCGTATTCGT CGACGAACTG CACGCCCTCG ACAAACAGCG CCTGTCCGGC AAGCTGTCCG 360  
AGGAGTTCAA CCGCGCCTAT ACCGGCATGT CCAGCGTGGT CAAAGCCACT GCCCGGCGCG 420  
45 TTGGCCGACT GGACGCCAG GCGCTGCAAA GCCAAGGCGT GCAGACGCTG CTCGAGGCCC 480  
ACCGCAACTG GAGCAAGCCC GAGCTGTGGT ACG 513

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## (2) INFORMATION FOR SEQ ID NO: 23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCCTCGGCAA ACAGCGCCTG TCCGGCAAGC 60  
TGTCCGAGGA GTTCAACCGC GCCTATACCG GCATGTCCAG CGTGGTCAAA GCCACTGCCC 120  
GGCGCGTTGG CCGACTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCGAG ACGCTGCTCG 180  
AGGCCCACCG CAACTGGAGC AAGCCCCGAGC TGTGGTACGC CATCGAGCGC GCCGGCAAGG 240  
TTTACACCTA CGATTACTAC CTGACCGCAC TGGATCTGGA GATGCACCCC GACGAGGGCA 300  
TCCAGGTGCG CCAGGACACG CAGATCTATC TGCAGCTGTA TTCCAAGACC CTGAACATGG 360  
CGCTGGTCAT CACCCTGCTC TGCGCCCTGC TCGGCTACCC GGTGGCCTAC TACCT 415

## (2) INFORMATION FOR SEQ ID NO: 24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 530 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCCTCGACAA ACAGCGCCTG TCCGGCAAGC 60  
TGTCCGAGGA GTTCAACCGC GCCTATACCG GCATGTCCAG CGTGGTCAAA GCCACTGCCC 120

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GGCGCGTTGG CCGACTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG ACGCTGCTCG 180  
 AGGCCCACCG CAACTGGAGC AAGCCCGAGC TGTGGTACGC CATCGAGCGC GCCGGCAAGG 240  
 5 TTTACACCTA CGATTACTAC CTGACCGCAC TGGATCTGGA GATGCACCCC GACGAGGGCA 300  
 TCCAGACCTG CCCGGGCGGC CGCTCGACCC CTATAGTGAG TAATCCCGCG GCCATGGCGG 360  
 CCGGGAGCAT GCGACGTCGG GCCCAATACG CCCTATAGTG AGTCGTATTA AAATTCACTG 420  
 10 GCCGTCGTTT TACAANGTNG TGAATGGNAA ANCCTGGCGT TACCCAACTT AATCGCCTTG 480  
 CAGCACATCC CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA GGCCCGCACC 530

15 (2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 319 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

30 CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCGTCGACAA ACAGCGCCTG TCCGGCAAGC 60  
 TGTCCGAGGA GTTGAACCGC GCGTATACCG GCATGTCCAG CGTGGTCAAA GCCAGTGCCC 120  
 GGCGCGTTGG CCGAGTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG AGCGCTGCTC 180  
 35 GAGGCCACCG GCAACTGGAG CAAGCCCGAG CTGTGGTACG CCATCGAGCG CGCCGGCAAG 240  
 GTTTACACCT AGGATTACTA CCTGACCGCA CTGGATCTGG AGATGCACCC CGACGAGGGC 300  
 40 ATCCAGACCT GCCCGGGCG 319

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 368 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

